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Unc45 Activates Hsp90-dependent Folding of the Myosin Motor Domain*[S]

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Myosin folding and assembly in striated muscle are mediated by the general chaperones Hsc70 and Hsp90 and involve a myosin-specific co-chaperone related to the *Caenorhabditis elegans* gene *unc-45*. Two *unc-45* genes are found in vertebrates, a general cell isoform, *unc45a*, and a striated muscle-specific isoform, *unc45b*. We have investigated the role of both isoforms of mouse Unc45 in myosin folding using an *in vitro* synthesis and folding assay. A smooth muscle myosin motor domain (MD) fused to green fluorescent protein (GFP) (MD::GFP) was used as substrate, and folding was measured by native gel electrophoresis and functional assays. In the absence of Unc45, the MD::GFP chimera folds poorly. Addition of either Unc45a or Unc45b dramatically enhances the folding in a reaction that is dependent on Hsp90 ATPase activity. Unc45a is more effective than Unc45b with a higher apparent affinity and greater extent of folding. The Unc45-Hsp90 chaperone complex acts late in the folding pathway and promotes motor domain maturation after release from the ribosome. Unc45a behaves kinetically as an activator of the folding reaction by stimulating the rate of the Hsp90-dependent folding by >20-fold with an apparent K_{act} of 33 nM. This analysis of vertebrate Unc45 isoforms clearly demonstrates a direct role for Unc45 in Hsp90-mediated myosin motor domain folding and highlights major differences between the isoforms in substrate specificity and mechanism.

There are over 18 classes of the protein myosin that form a large family of actin-based motors and power a variety of motility-based cellular functions (1). All myosin classes share a conserved catalytic domain of ~750 residues that contains the ATPase activity and actin-binding site responsible for force production and motion (2). We have shown that folding and assembly of the class II vertebrate striated muscle myosin follow a chaperone-assisted pathway (3, 4). In a reticulocyte lysate synthesis assay, the myosin rod domain assembles and dimerizes, and the myosin light chains associate with the motor domain, but myosin motor domain folding is slow and incomplete. The striated muscle motor domain is folded efficiently only in muscle cells, indicating a requirement for muscle-specific factors (3).

In striated muscle cells myosin maturation and assembly proceed through the formation of a transient complex containing nascent myosin with partially folded motor domains assembled into filaments and associated with the general chaperones Hsp90[2] and Hsc70 (5–7). Striated muscle myosin transits through this chaperone complex on the pathway to myofibril assembly. The general chaperones Hsp90 and Hsp70 are abundant proteins in muscle and in the reticulocyte lysate synthesis system (8), yet this system is not sufficient for the folding of the striated muscle myosin motor domain. What other factors are required for efficient motor domain folding?

The *Caenorhabditis elegans unc-45* gene encodes an ~103-kDa protein (UNC-45) that is important for the establishment of organized body wall muscles (9–11). The protein has three basic motifs as follows: an N-terminal domain with three tetratricopeptide repeats (TPR), a central region of unknown function, and an ~450-residue C-terminal UCS domain that is shared by proteins that interact with myosin. The TPR motif is a protein-protein interaction module of 34 amino acids that is often involved in Hsp90 and Hsp70 binding (12); *C. elegans* UNC-45 interacts with Hsp90 via the TPR motif (13). The UCS domain is a homology region found in *C. elegans* *unc-45*, in CRO1 from the filamentous fungus *Podospora anserina* (14) and in *S. cerevisiae* She4 (15), the three founding family members. Mutations in the UCS domain of these genes result in decreased accumulation and altered assembly of striated muscle myosin filaments, disruption of contractile ring formation, and disorganization of the actin cytoskeleton, linking them to myosin cellular activities. Rng3p, the *Schizosaccharomyces pombe* UCS protein, activates myosin II motility *in vitro*, suggesting a direct interaction and regulatory role for the UCS domain (16). The UCS domain of UNC-45 blocks thermal aggregation of the myosin head consistent with a role as a co-chaperone that links Hsp90 with the motor domain (13). Swo1p, the *S. pombe* Hsp90 ortholog, genetically interacts with Rng3p to facilitate myosin assembly in the contractile ring again linking these three proteins despite the absence of the TPR motif in Rng3p (17, 18).

There is a single *unc-45* gene in *C. elegans* and *Drosophila*. Temperature-sensitive alleles of this gene disrupt thick fila-

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[2] The abbreviations used are: Hsp, heat shock protein; MD, motor domain; MD::GFP, chimera of myosin motor domain fused to green fluorescent protein; TPR, tetratricopeptide repeat.

ment assembly in the body wall muscle at nonpermissive temperatures, and UNC-45 co-localizes with non-muscle myosin in *C. elegans* embryos (9, 11, 19, 20). Vertebrates express two distinct genes that are orthologs of *C. elegans unc-45* (21). One, *unc45a* (formerly GC-UNC-45), is expressed generally in all tissues, and expression of the other, *unc45b* (formerly SM-UNC-45), is limited to striated muscle. We have cloned and expressed the two isoforms of mouse Unc45 to assess the role of these proteins in myosin motor domain folding. Unc45a and Unc45b both stimulate the folding of a smooth muscle myosin motor domain in the reticulocyte lysate synthesis assay. Unc45a shows selectivity for the smooth muscle myosin motor domain and stimulates folding by activating the rate of the Hsp90-dependent reaction. This is the first direct demonstration of Unc45 chaperone activity in the *de novo* folding of the myosin motor domain and clearly establishes a role for Unc45a and Hsp90 in myosin maturation. Surprisingly, addition of neither Unc45 isoform is sufficient to activate the folding of the striated muscle motor domain indicating that additional factors are still required for this folding pathway.

EXPERIMENTAL PROCEDURES

Vector Construction—Full-length cDNA for Unc45a and Unc45b were cloned by reverse transcription-PCR from NIH 3T3 fibroblasts and mature C2C12 myotube total RNA using primers derived from the mouse genome data base. The cloned cDNAs were validated by bidirectional sequencing and comparison to the mouse genome. The inserts were cloned into the NcoI-NotI sites of pET21d (Novagen, Darmstadt, Germany) after modification of the 5′ and 3′ ends to incorporate these sites. The six His tag coding sequence in the vector was added to the 3′ end of the cDNAs with the addition of a two-residue linker sequence. Similarly, a 3× FLAG tag was substituted for the His tag in the same base vector for synthesis of C-terminal FLAG-tagged variants of the Unc45 isoforms.

Construction of the vectors for the skeletal muscle MD::GFP chimera (Sk_{795}GFP) has been described in detail elsewhere (3, 4). The cDNA encoding the chicken gizzard smooth muscle myosin motor domain was provided by Kathy Trybus, University of Vermont (22). The design of smooth muscle MD::GFP expression vector is identical to the Sk_{795}GFP vector with the junction between the MD and GFP at a conserved sequence in an α-helix preceding the essential LC-binding site (supplemental Fig. 2s). The skeletal muscle motor domain was excised and replaced with the smooth muscle motor domain, and the construction was confirmed by bidirectional sequencing. The smooth muscle MD::GFP chimera produced a 116-kDa protein when expressed in the coupled translation assay.

Protein Preparation—The pET21-Unc45a-His and pET21-Unc45b-His vectors were transformed into *Escherichia coli* BL21 (DE3) Codon Plus, and single colonies were grown aerobically at 37 °C in ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml) supplemented LB medium to an OD of 0.4 and then induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside and harvested 4–6 h later. Bacterial lysis and purification of Unc45-His were done with a nickel-nitrilotriacetic acid fast start kit following the manufacturer's instructions for native proteins (Qiagen Inc., Valencia, CA). Proteins bound to the affinity resin were eluted with a buffer containing 250 mM imidazole, pooled, and dialyzed against 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, and 25 mM Tris-HCl, pH 8.0. The pooled protein was applied to a Tricorn Mono Q 10/100 GL column and eluted with a linear 0.15–1.0 M NaCl gradient. The Unc45-containing fractions were pooled, concentrated, and further purified by gel filtration on a Superose 6 10/300 GL column (GE Healthcare). This preparation was >98% pure, and the protein concentrated with an Amicon Ultra 4 centrifugal filter device (Millipore) was stored frozen in Mono Q buffer (supplemental Fig. 1s). The FLAG-tagged variants of Unc45 were expressed using the pET system and purified by ion-exchange and gel filtration chromatography, omitting the nickel-nitrilotriacetic acid purification step.

Hsp90 was purified from rabbit reticulocyte lysate (Green Hectares, Madison, WI). A high salt stripped, ribosome-free supernatant was prepared from the lysate by adding KCl to 1 M and centrifugation for 61 min at 70,000 rpm in a Beckman Ti70 rotor at 4 °C. The supernatant was dialyzed into 100 mM KCl, 0.1 mM EDTA, 20 mM Tris-Cl, pH 7.5 (Buffer A), and fractionated by ion-exchange chromatography on a Tricorn Mono Q 10/100 GL column (GE Healthcare) with a linear 0.1–0.5 M KCl gradient. Hsp90 elutes at ~0.4 M KCl. The Hsp90 fractions were pooled, concentrated with an Amicon Ultra 4 centrifugal filter, and stored frozen in Buffer A. Myosin and myosin subfragments were prepared from adult white leghorn chicken pectoralis muscle as described previously (23).

Assays—All *in vitro* expression vectors contain myosin coding sequences cloned downstream of the SP6 promoter of the pGEM4 vector (Promega, Madison, WI). Coupled transcription and translation assays using Promega Quick Mix SP6 kits were supplemented with Redivue L-[^{35}S]methionine (GE Healthcare) and incubated for 90 min at 30 °C with 2 μg of plasmid DNA per 50-μl reaction as described previously (3). Folding of the MD::GFP chimera was analyzed by native gel electrophoresis in a modified Laemmli Tris-glycine electrophoresis system that lacked SDS (24). The stacking gel is 5% acrylamide in 62.5 mM Tris-HCl, pH 6.8, and the running gel is 10% acrylamide in 375 mM Tris-HCl, pH 8.8. The running buffer is 25 mM Tris-HCl, 192 mM glycine, pH 8.3, and sample loading buffer is 50 mM Tris-HCl, pH 8.0, 10% glycerol, and 0.01% bromphenol blue. Electrophoresis was for 3 h at 20–25 mA constant current (4 °C). Band intensities were quantified from autoradiographs of dried gels with iVision image analysis software (BioVision Technologies, Exton, PA). Proteins were also analyzed by SDS-PAGE (24).

Anti-FLAG M2 monoclonal antibody-coupled agarose beads were washed according to the manufacturer's recommendations (Sigma), and Unc45^{FLAG} proteins were incubated with 1:1 slurry of M2-agarose beads overnight at 4 °C. The anti-FLAG-agarose beads with bound protein were collected by brief centrifugation at 1,000 × *g* and washed with 1 ml of buffer (0.15 M NaCl, 50 mM Tris-Cl, pH 7.4) for 40 min at 4 °C, followed by three washes with the same buffer for 10 min each before use in binding assays. The beads were incubated with increasing amounts of Hsp90 (0–4 μM) for 2 h at 4 °C, recovered by centrifugation, and washed four times with buffer. Bound proteins were extracted in SDS-PAGE sample buffer and analyzed.

Hsp90 and Unc45 binding interactions were analyzed on a Superose 6 HR 10/300 gel filtration column (GE Healthcare) in Buffer A. The Superose 6 column was calibrated using purified proteins as follows: rabbit Hsp90, Unc45a, Unc45b, and papain myosin subfragment-1. Actin binding assays used 25 μl of a translation reaction and 50 μg of fresh F-actin depleted of ATP or with added MgATP as described previously (3).

Motor domain folding kinetics were measured by translating the smooth MD::GFP chimera for 90 min at 30 °C and terminating translation with 0.36 μM cycloheximide (Sigma). Aliquots of the translation were incubated with varying concentrations of Unc45a, and samples were removed at time points and diluted into native gel loading buffer containing 7 μM geldanamycin (A. G. Scientific Inc., San Diego, CA) to inhibit Hsp90 ATPase activity.

RESULTS

Motor Domain Folding Assay—The two mouse orthologs of *C. elegans unc-45* were cloned by reverse transcription-PCR from mouse NIH 3T3 fibroblast (Unc45a) and C2C12 myotubes (Unc45b) (supplemental Fig. 1s). The full-length cDNA clones were inserted into bacterial expression vectors and modified by addition of a C-terminal six His tag sequence. Both mouse Unc45 isoforms were expressed in *E. coli*, extracted as soluble proteins, and affinity purified via the His tag. Analysis of purified Unc45a and Unc45b by SDS-PAGE demonstrates >98% purity, and they elute as ~100 kDa monomeric species when analyzed by size-exclusion chromatography.

We have demonstrated the utility of *in vitro* synthesis of myosin subfragments in a rabbit reticulocyte lysate-coupled transcription and translation assay for studying the steps in folding and maturation of myosin (3, 4). Light chain binding and folding and dimerization of the S2 domain of the rod were shown to precede motor domain folding. Furthermore, using a striated muscle motor domain GFP chimera (MD::GFP), we have found that folding of the muscle MD is dependent on factors uniquely present in muscle cells (3). In contrast to striated muscle myosin, smooth muscle myosin has been efficiently produced and isolated from heterologous expression systems (22). We constructed an MD::GFP chimera of the smooth muscle myosin that is identical in design to the striated muscle MD::GFP chimera (supplemental Fig. 2s). This smooth muscle myosin chimera has been used to develop a *de novo* synthesis and folding assay and to test the effect of the putative myosin co-chaperones Unc45a and Unc45b on myosin motor domain folding.

The smooth MD::GFP chimera is efficiently synthesized in the reticulocyte lysate producing a 116-kDa band when analyzed by SDS-PAGE (Fig. 1*A*). However, analysis by native gel electrophoresis shows only limited folding of the motor domain. If the lysate is supplemented with either Unc45a or Unc45b, there is a dramatic increase in radioactivity migrating as a discrete band in the native gel. The position of the discrete band corresponds to the migration of native myosin subfragments in this gel system (not shown). A densitometric scan of the native gel shows that in the absence of Unc45 the bulk of the radioactivity migrates as a series of diffuse bands near the top of the gel, and only a small fraction (<10%) migrates in the position of the native protein (Fig. 1*B*). The inclusion of Unc45a in the synthesis reaction shifts the bulk of the slowly migrating radioactivity near the top of the gel to the faster migrating discrete band. The level of MD::GFP synthesis is unaffected by addition of the purified Unc45 as shown by SDS-PAGE of the reactions. The increased intensity is not a consequence of increased synthesis or enhanced stability of the MD::GFP synthesized in the supplemented reactions but rather a shift to a more uniform conformation.

Analysis of folding reactions by gel filtration chromatography demonstrates the appearance of the discrete band in the native gel coincides with the accumulation of MD::GFP monomers with hydrodynamic properties similar to striated muscle myosin S1, an active proteolytic myosin subfragment (Fig. 1*C*). The protein in the peak fractions from the Unc45a supplemented reaction corresponds to the faster migrating discrete band detected in the native gel. Finally, this species exhibits ATP-sensitive actin binding activity, confirming that it is indeed the native conformation (Fig. 1*D*). Therefore, the addition of either Unc45a or Unc45b enhances myosin motor domain folding, and this is readily quantified by native gel electrophoresis.

Unc45 Requirement for Myosin Motor Domain Folding—Titration of the MD::GFP folding reaction with both Unc45 isoforms demonstrates that Unc45a enhances folding to a greater extent and at a lower concentration than Unc45b (Fig. 2). This is readily apparent if the concentration dependence of the folding reaction is treated as a simple hyperbolic binding curve. The apparent dissociation constant for Unc45a (K_D = 19 nM) is less that half that for Unc45b (K_D = 53 nM). The extent of the motor domain folding also is different for the two isoforms. Up to 80% of the radioactivity is converted to the native conformation at saturating concentrations of Unc45a, compared with less than 50% with Unc45b. Folding is essentially complete when Unc45a is included in the reaction because incomplete chains due to internal initiation and premature termination account for much of the remaining 20% of the non-native radioactivity in this assay.

Hsp90 has been identified as a chaperone on the myosin maturation pathway in striated muscle, and Unc45a has been shown to interact with Hsp90 (5, 6, 13, 25). To determine whether Hsp90 has a role in myosin motor domain folding, the ATPase activity of Hsp90 was specifically inhibited by the ansamycin antibiotic geldanamycin (26). Inhibition of Hsp90 activity completely blocks the stimulation of motor domain folding by Unc45a and Unc45b (Fig. 3*A*). Even the basal level of motor domain folding detected in the absence of added Unc45 is inhibited by geldanamycin, indicating all motor domain folding in this assay is Hsp90-dependent. The synthesis and stability of the MD::GFP chimera in the reticulocyte lysate were not affected by geldanamycin; so inhibition of Hsp90 activity is not accelerating motor domain turnover. Furthermore, geldanamycin has no effect on stability of the folded state. These results show that Unc45-enhanced MD::GFP folding is dependent on Hsp90 ATPase activity. Western blots with antibodies to either Unc45a or Unc45b do not reveal detectable levels of endoge-

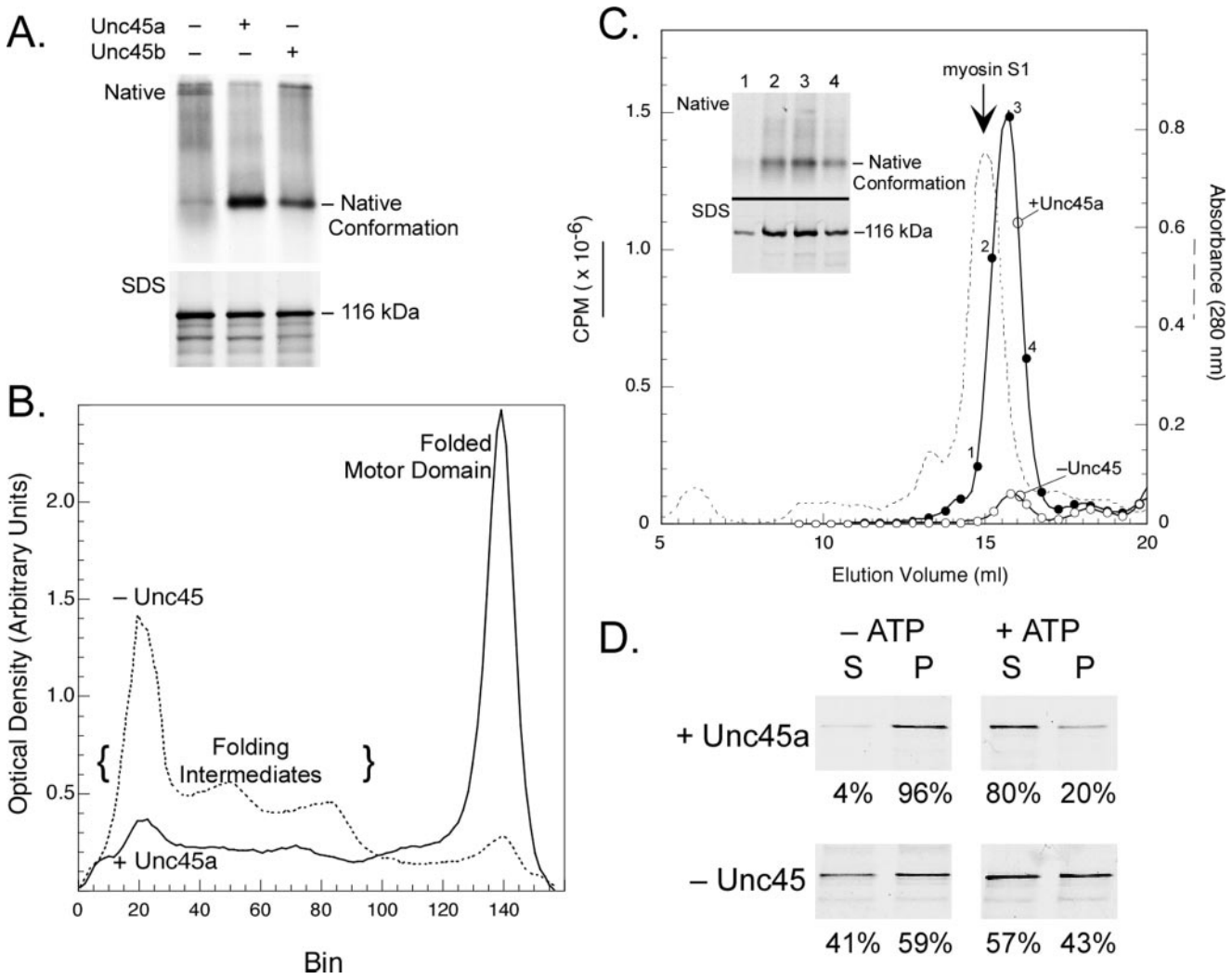

FIGURE 1. **Native gel electrophoresis and SDS-PAGE of the smooth muscle myosin MD::GFP chimera synthesized in the absence and presence of Unc45a and Unc45b.** *A,* native gel resolves the unfolded motor domain as a diffuse smear near the top of the gel, and the folded motor domain as a discrete band (*Native Conformation*). Addition of Unc45a or Unc45b to the synthesis assay dramatically enhances the formation of the discrete band. The SDS gel shows that the amount of MD::GFP synthesized is not affected by addition of Unc45. *B,* a densitometric scan of the native gel shows that the unfolded motor domain migrates as a broad and heterogeneous population of intermediates when synthesized in the absence Unc45 (−*Unc45*). Addition of Unc45a converts this radioactivity to a discrete band (+*Unc45a*). *C,* analysis by gel filtration chromatography of the smooth muscle MD::GFP synthesized in the presence of added Unc45a shows that it elutes as a mono-disperse peak (*solid line* with *filled circles*) at a position consistent with a MD::GFP monomer. Electrophoresis of the protein in the peak column fractions on a native gel (*inset*) confirms that the discrete band is the native conformation. The elution position of the 135-kDa myosin subfragment-1 (*dotted line*) is shown for comparison. *D,* SDS-PAGE of radioactive MD::GFP bound to F-actin in a sedimentation assay. The folded MD::GFP pellets (*P*) with F-actin in the absence of ATP (−) and is released in the supernatant (*S*) when ATP (+) is added. The percent of the total radioactivity for each condition is noted *below* each lane. The MD::GFP chimera synthesized in the presence of Unc45a has ATP-sensitive actin binding activity that is characteristic of an active myosin subfragment.

nous Unc45 in the reticulocyte lysate (data not shown). This suggests that Unc45 might stimulate motor domain folding by accelerating delivery of substrate to Hsp90.

Hsp90 is generally considered a late chaperone regulating the maturation of macromolecules and activation of multiprotein complexes (27). In the preceding experiments, Unc45 was included during the synthesis of the myosin motor domain. To determine the timing of the interaction of Unc45 in the motor domain folding pathway, we separated synthesis from folding by first translating the MD::GFP and then inhibiting protein synthesis before adding the Unc45 (Fig. 3*B*). The extent of MD::GFP folding was the same whether Unc45 was present during translation or added post-translation. It is apparent that the interaction of Unc45 and Hsp90 with the unfolded motor domain is a late step in the folding pathway.

Unc45a Stimulates the Rate of the Folding Reaction—The observations that Unc45a acts after synthesis and that folding can be completely blocked by inhibition of Hsp90 ATPase activity provide the tools needed to examine the kinetics of the folding reaction (Fig. 4). The MD::GFP chimera was synthesized in the absence of Unc45a and the synthesis stopped with cycloheximide. Aliquots of the unfolded MD::GFP were incubated with various concentrations of Unc45a. Time points of the reactions were sampled, and folding was stopped by inhibiting Hsp90 with geldanamycin. The initial rates of the accumulation of the folded MD::GFP were then determined and analyzed. In this assay, Unc45a behaves as an activator of the Hsp90-dependent folding activity. The K_{act} of Unc45a (33 nM) from the folding kinetics is very similar to the apparent K_D (19 nM) determined from the end point titration (Fig. 2). The

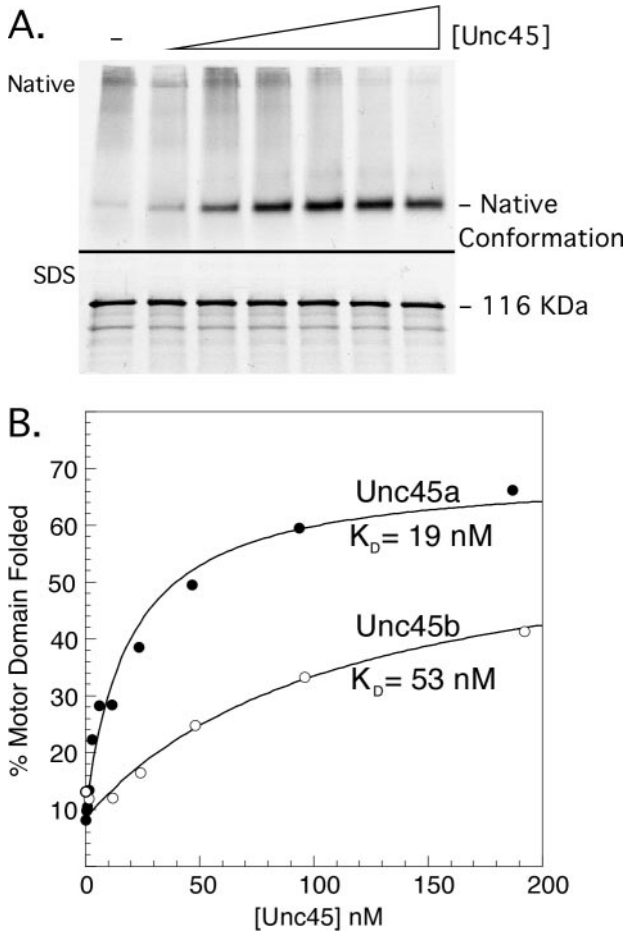

FIGURE 2. **The Unc45 concentration dependence of smooth muscle MD::GFP folding.** Varying concentrations (0–200 nM) of Unc45a or Unc45b were added during synthesis of smooth muscle MD::GFP, and aliquots of the radioactive protein were analyzed by electrophoresis. *A,* native and SDS-PAGE of MD::GFP folding assay (− indicates no added Unc45). The extent of folding was measured from the distribution of radioactivity in the *Native* band position relative to the sum of all other intermediates. *B,* MD::GFP folding has a hyperbolic dependence on Unc45 concentration that was treated as a simple binding interaction. Unc45a has a higher folding activity as reflected by the lower apparent K_D and greater extent of folding over the range of concentration tested.

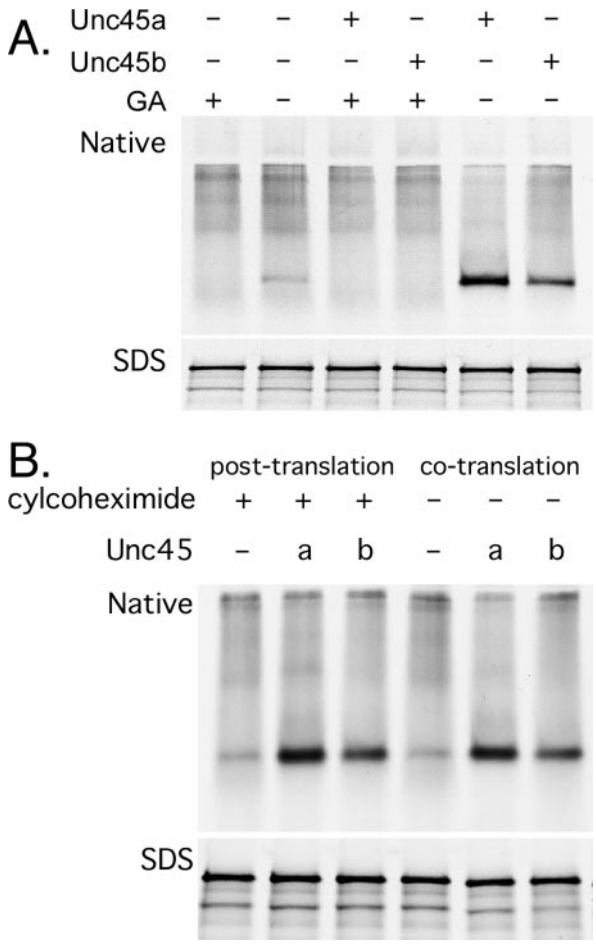

FIGURE 3. **Hsp90 ATPase activity is required for motor domain folding, and Unc45 and Hsp90 act post-translation.** *A,* addition of the Hsp90 inhibitor geldanamycin (*GA*) to the synthesis assay completely blocks the Unc45-mediated enhancement of the smooth muscle MD::GFP folding. Hsp90 activity was inhibited with 7 μM geldanamycin. Despite the strong effect on MD folding, the SDS-PAGE shows that geldanamycin does not affect the synthesis or stability of the MD::GFP. *B,* Unc45/Hsp90 chaperones act late in the folding pathway. Unc45-enhanced MD::GFP folding was the same whether it was present during the 90-min translation (co-translation) or added after translation was terminated with cycloheximide (post-translation). Cycloheximide was added after 90 min of synthesis, and then Unc45a of Unc45b were added, and incubation was continued for an additional 90 min.

rate of the MD::GFP folding reaction is accelerated >20-fold at saturating Unc45a concentrations.

These data are consistent with a mechanism where Unc45a acts as an activator of Hsp90 folding activity, but it is not clear whether the activation constant reflects the binding interaction between Hsp90 and Unc45a or between Unc45a and the unfolded MD::GFP substrate. To address this question, we analyzed the binding interaction between both isoforms of Unc45 and Hsp90 in solution. Hsp90 was incubated with Unc45a or Unc45b, and the reactions were analyzed by gel filtration chromatography (Fig. 5, *A* and *B*). Unc45a and Unc45b both elute from a Superose 6 column in a single symmetric peak at an elution volume consistent with a monomer of ~100-kDa apparent mass. In contrast, Hsp90 elutes earlier from the column in a broad double peak consistent with its larger dimeric mass (~180 kDa) and heterogeneous conformation (28).

The formation of a complex between Unc45 and Hsp90 changes the elution profile of the individual components. This is readily apparent for the complex formed by Unc45b and Hsp90 (Fig. 5*B*). There is a decrease in the Unc45b monomer peak height, an increase in height of the peak eluting in the position of Hsp90, and a sharpening of the peak into a more symmetric shape. This is consistent with the shift of Unc45b into a complex with Hsp90 that has a more compact conformation than the pure Hsp90 dimer. The Unc45a shows considerably less evidence of binding to Hsp90 (Fig. 5*A*). There are slight shifts in the Hsp90 and Unc45a peaks consistent with a weak binding interaction, but these changes are dramatically less than those observed with Unc45b.

Analysis of the integrated density of the bands on SDS-PAGE of the column fractions provides an estimate of the binding stoichiometry and affinity (Fig. 5, *C* and *D*). Less than 10% of the Unc45a elutes with the Hsp90, and the Hsp90/Unc45a ratio across the region of overlap is high, consistent with a low binding affinity. In contrast, over 40% of the Unc45b co-elutes with the Hsp90, and the Hsp90/Unc45 ratio in the center of the overlap approaches a value of 2. Because Hsp90 is a dimer, that

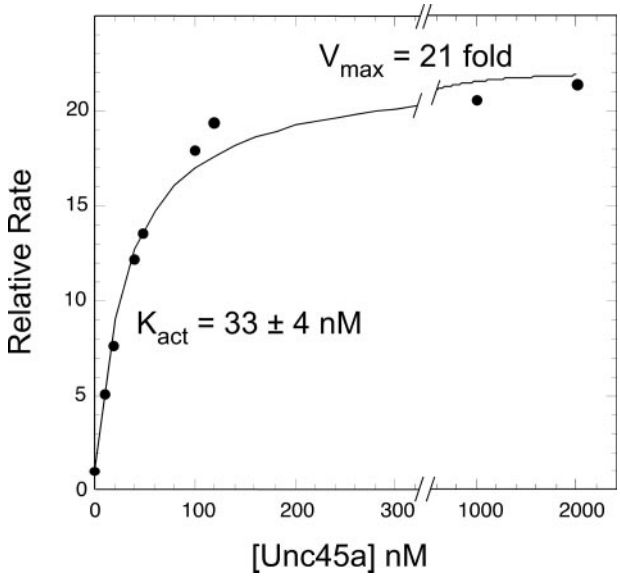

FIGURE 4. **Unc45a stimulates the rate of motor domain folding over 20-fold.** The smooth MD::GFP chimera was synthesized for 90 min and then cycloheximide was added to terminate translation. Aliquots of the lysate were supplemented with various concentrations of Unc45a and incubated at 22 °C. The MD folding was terminated by adding geldanamycin to inhibit Hsp90, and the extent of MD::GFP folding was measured by native gel electrophoresis. The rate of accumulation of MD::GFP was linear for the first 20 min of the folding reaction for most concentrations of Unc45a tested. This initial rate relative to the rate measured in the lysate without added Unc45a is plotted. Unc45a accelerated the folding reaction by >20-fold, and the concentration dependence of the relative folding rates fit the data with an apparent activation constant, $K_{act} = 33 \pm 4$ nM.

ratio would correspond to a 1:1 molar complex formed between Hsp90 and Unc45b.

Based on the elution data there is significant difference in Hsp90 binding affinity between Unc45a and Unc45b. This is confirmed in a pulldown assay that measures the concentration dependence of Hsp90 binding to immobilized Unc45 isoforms (Fig. 6). The apparent K_D value for Hsp90 binding to Unc45b (0.5 μM) is 15-fold stronger than to Unc45a (7.8 μM). So although both co-chaperones enhance the Hsp90-dependent folding reaction, Unc45a and Unc45b differ significantly in Hsp90 binding affinity. In addition, these results show that Unc45a binds Hsp90 over 2 orders of magnitude weaker in the absence of substrate protein (MD::GFP) than the measured K_{act} (33 nM) for the folding reaction.

Finally, is Unc45b the missing muscle-specific factor required for efficient striated myosin motor domain folding? Unc45a and Unc45b are active co-chaperones that enhance Hsp90-dependent folding of the smooth muscle motor domain. However, neither Unc45a nor Unc45b is sufficient to complement the folding of the striated muscle myosin motor domain (Fig. 7). This suggests that other factors not present in the reticulocyte lysate are still required for folding of the striated motor domain. The factor(s) may act before or in concert with the late Unc45/Hsp90 folding components. The striated muscle myosin MD::GFP design used here is correctly folded when expressed in striated muscle (3), so this assay will be a valuable tool to test additional factors isolated from muscle for a role in this more complicated folding pathway.

DISCUSSION

Unc45 Activates an Hsp90-dependent Folding Pathway—We have analyzed the role of the Hsp90 co-chaperone protein Unc45 in myosin folding and demonstrated that a primary activity of Unc45 is to activate the Hsp90-dependent folding of the myosin motor domain. Both vertebrate isoforms of Unc45, the generally expressed Unc45a and striated muscle-specific Unc45b, enhance the *de novo* folding of a smooth muscle myosin motor domain in a reaction that is dependent on Hsp90 ATPase activity. Selectivity for the smooth muscle myosin substrate and striking differences in Hsp90 binding affinity are demonstrated for the two different Unc45 isoforms. This suggests mechanistic differences in the folding pathways supported by the vertebrate Unc45 isoforms. This conclusion is supported by the observation that, although both isoforms are active in folding the smooth muscle myosin, neither is sufficient for folding the striated myosin motor domain.

The Unc45/Hsp90 chaperones act late during motor domain folding after translation termination on a heterogeneous population of folding intermediates that are converted to the native conformation. Unc45a stimulates the rate of motor domain folding over 20-fold with an apparent activation constant of 33 nM. Unc45a might act as an activator of the Hsp90-dependent folding reaction by targeting the motor domain to the chaperone machinery. We do not detect Unc45 protein in the reticulocyte lysate with antibodies to either Unc45a or Unc45b (data not shown). Therefore, the low level of motor domain folding in the unsupplemented lysate corresponds to the basal activity of Hsp90. The complete inhibition of motor domain folding by an Hsp90 ATPase inhibitor confirms this conclusion and is consistent with a mechanism whereby Unc45 facilitates folding by targeting substrate to the chaperone machinery. This result also links Hsp90 for the first time to the maturation of smooth muscle myosin.

Functional Differences between the Unc45 Isoforms—The general cell Unc45a isoform shows selectivity for the smooth muscle myosin motor domain with a higher apparent binding affinity and a greater extent of folding in this assay than the muscle-specific isoform. Unc45a is expressed in smooth muscle and was cloned from these cells as SMAP-1 (smooth cell muscle associated protein 1, direct submission GenBank™ accession number AB014736). The selectivity might reflect the co-evolution of the smooth muscle myosin with a cognate co-chaperone. Substrate selectivity adds support to the hypothesis that one function of Unc45 is to target substrate to the general chaperone machinery as a necessary step for efficient motor domain folding.

Substrate selectivity is not the only difference between Unc45a and Unc45b. The muscle-specific Unc45b isoform has a significantly higher affinity for Hsp90 than the general Unc45a isoform. In a related study, we have found that the endogenous Unc45b in muscle cytosol, as well as overexpressed recombinant Unc45b, isolated as a complex with Hsp90 (29). Consequently, we were surprised by the striking difference in Hsp90 binding affinity between the two isoforms. The activation constant for Unc45a stimulation of motor domain folding ($K_{act} = 33$ nM) is much lower than the apparent binding affinity

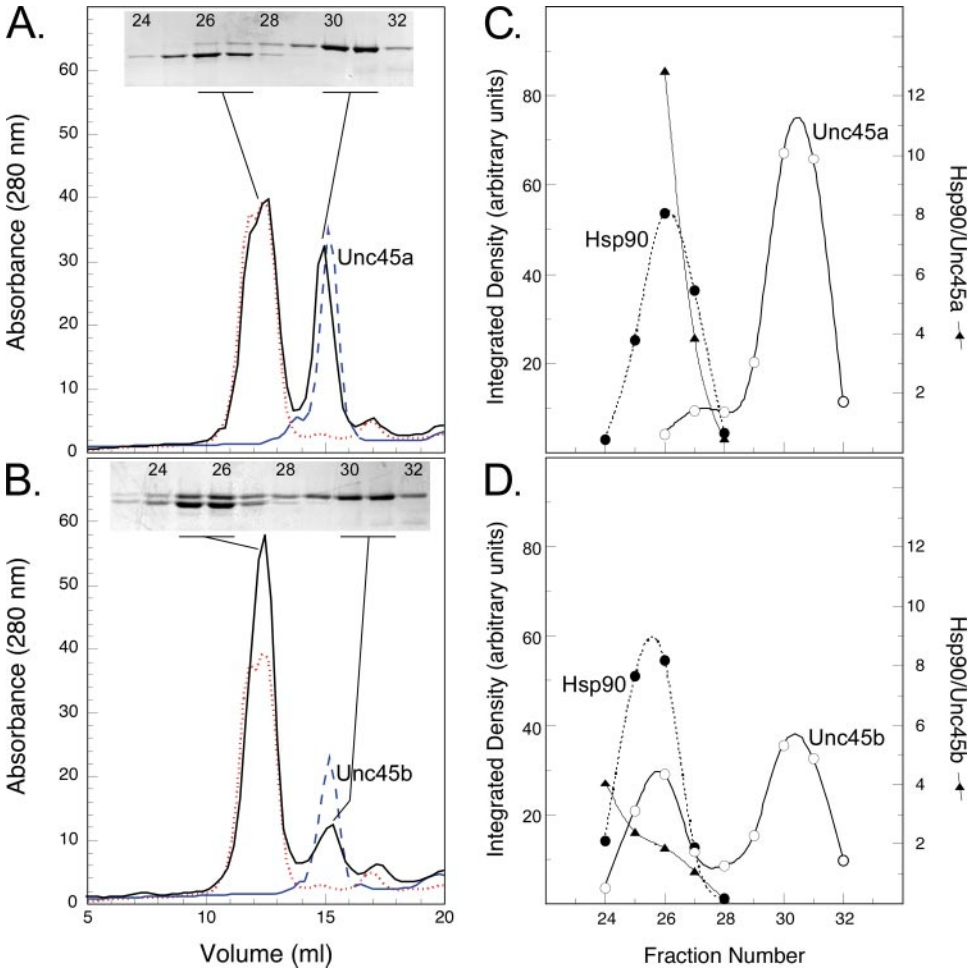

FIGURE 5. **Solution binding of Unc45a and Unc45b to Hsp90 was measured by gel filtration chromatography.** Unc45a (100 μg) or Unc45b (60 μg) were incubated with Hsp90 (100 μg) at 22 °C for 30 min in a final volume of 200 μl and then analyzed by gel filtration. *A,* elution profile (absorbance at 280 nm) for binding reaction of Unc45a with Hsp90 (*heavy solid line*) is compared with the elution profile of pure Hsp90 (*dotted red line*) and pure Unc45a (*dashed blue line*). The *inset* shows the SDS-PAGE analysis of the column fractions. Weak binding of Unc45a to Hsp90 is detected by the change in shape of the peaks and presence of both proteins in the peak fractions in the SDS gel. *B,* elution profile for binding reaction of Unc45b with Hsp90 (*heavy solid line*) is similarly compared with the elution profile of pure Hsp90 (*dotted red line*) and pure Unc45b (*dashed blue line*). The decrease in the Unc45b peak height and the increase in peak height and sharpening of the peak eluting in the Hsp90 position demonstrate complex formation. The *inset* confirms the co-elution of Unc45b and Hsp90. *C,* densitometry of the bands in the SDS gel for the Unc45a and Hsp90 column fraction shows that ~10% of the Unc45a co-elutes with Hsp90. This is confirmed by the plot of the Hsp90:Unc45a density ratios. *D,* stronger binding of the Unc45b to Hsp90 is confirmed by the densitometry of the SDS gel of the column fractions. The ratio of Hsp90:Unc45b in fractions 25–27 approaches 2, a value that would correspond to a 1:1 complex of Hsp90 dimer with Unc45b monomer.

of Unc45a for Hsp90. The weak binding interaction with Hsp90 suggests that the activation constant might reflect the binding of Unc45a to the unfolded motor domain. Alternatively, both Unc45a and the unfolded motor domain may be required for a high affinity binding interaction with Hsp90. We suspect that this ternary complex is a transient intermediate formed as unfolded substrate is transferred to the chaperone machinery and folded. This aspect of the mechanism clearly needs further work. Nonetheless, these results identify key differences between the two vertebrate Unc45 isoforms.

Requirements for Smooth Versus Striated Muscle Myosin II Folding—An even more striking difference is demonstrated between the folding pathway of the smooth and the striated muscle myosin motor domains. Neither Unc45a nor Unc45b is sufficient to promote the folding of the striated muscle myosin motor domain under conditions that support nearly complete folding of the smooth muscle myosin motor domain. The muscle-specific Unc45b is active in folding of the smooth muscle myosin motor domain, so the co-chaperone activity and the correct isoform for folding the striated motor domain are present in the assay. Nonetheless, the striated motor domain is not folded. We have shown that the striated myosin MD::GFP chimera assayed here is correctly folded only when expressed in muscle cells (3). So, other muscle-specific factors in addition to Unc45b are required for efficient folding of striated muscle myosin.

Unc45 and Hsp90 are late chaperones acting after translation. Perhaps early muscle-specific chaperones are required for delivery of the striated myosin motor domain in a folding competent conformation to the late chaperone machinery. Unc45b and Hsp90 bind the unfolded striated muscle motor domain synthesized in the reticulocyte lysate, so the conformation present in the lysate is recognized by the late chaperone machinery (29). However, this conformation might be bound but is still not competent for maturation. Alternatively, other factors may be required to regulate the dissociation of the MD/Unc45b/Hsp90 interaction to complete folding. The higher affinity of Unc45b for Hsp90 might reflect a mechanistic requirement for a dissociation factor that is not required for the weaker binding Unc45a isoform. These differences in the folding pathway clearly reflect the difficulty that has been associated with the expression of the striated muscle myosin family in heterologous systems (3). The assay developed here should assist in searching for missing factors that are necessary to complete the striated muscle myosin folding pathway.

Unc45, Hsp90, and Myosin Filament Assembly—Myosin maturation and assembly in striated muscle proceeds through the formation of a transient complex containing partially folded myosin assembled into filaments and associated with Hsp90 and Hsc70 (5). Genetic interactions suggest a fundamental role for Unc45 and Hsp90 in myosin filament assembly. In *C. elegans*, temperature-sensitive alleles of *unc-45* disrupt thick filament assembly in the body wall muscle at nonpermissive temperature (9, 11). In the zebrafish embryo the *unc45b* gene is

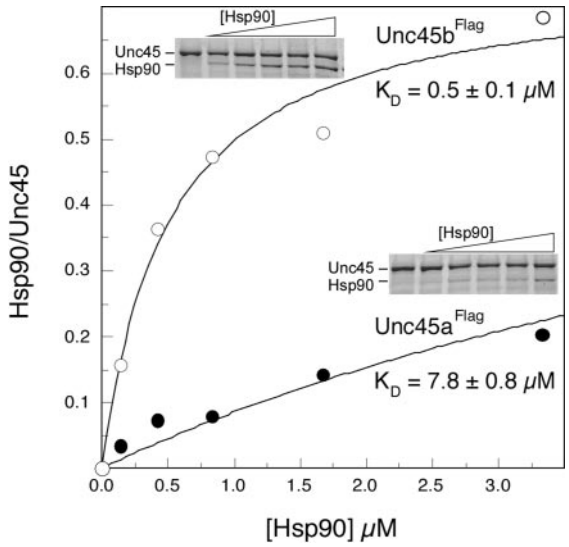

FIGURE 6. **Binding assay of Unc45a^{FLAG} and Unc45b^{FLAG} to Hsp90.** Unc45a^{FLAG} and Unc45b^{FLAG} (2 μg each) were bound to anti-FLAG M20-agarose beads (10 μl) and washed to remove excess protein. The beads were incubated with increasing amounts of Hsp90 (0–4 μM) for 2 h at 4 °C. The beads were recovered by centrifugation and washed and then suspended in SDS-PAGE sample buffer, and the bound proteins were analyzed. The *insets* show the SDS-PAGE lanes of the bound fraction in the pulldown assay. The binding curves plot the ratio of Hsp90:Unc45 bound to the beads and were fit to a hyperbolic binding curve with an empirical Hsp90:Unc45b binding ratio of 0.75. Unc45a binds Hsp90 over 15-fold weaker than Unc45b. The maximum binding ratio observed reflects the available Unc45^{FLAG} on the beads and not the binding stoichiometry of Hsp90 and Unc45. Both Unc45a^{FLAG} and Unc45b^{FLAG} have the same activity and selectivity as the His-tagged counterparts in the synthesis and folding assay with the smooth muscle myosin MD::GFP substrate (data not shown).

expressed exclusively in striated muscle (30). Targeted knockdown of Unc45b protein in zebrafish results in paralysis and cardiac dysfunction correlated with a loss of myosin filaments in sarcomeres, consistent with a role for Unc45b in folding of muscle-specific myosin isoforms. A striated muscle-specific knockdown of Hsp90α1 in zebrafish produces a complementary phenotype and is accompanied by an up-regulation of Unc45b expression and decreased myosin stability (6, 7). We have shown here that Unc45 is an active component of the chaperone machinery that mediates myosin motor domain folding providing a biochemical mechanism for the interaction between Unc45 and Hsp90 in myosin filament assembly. However, for striated muscle myosin folding additional factors are required to the complete the pathway.

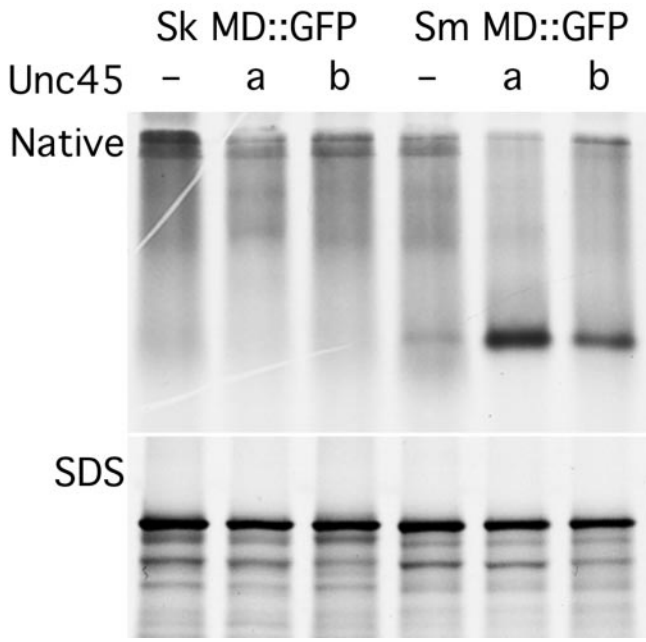

FIGURE 7. **Neither Unc45a nor Unc45b complements the folding of the striated muscle myosin MD::GFP chimera in the synthesis assay.** Skeletal muscle myosin MD::GFP chimera (Sk MD::GFP) and smooth muscle myosin MD::GFP (Sm MD::GFP) were synthesized without Unc45 (*lane* −) or with added Unc45 (*lanes a* or *b*). Folding of the smooth muscle MD::GFP chimera is stimulated by Unc45. Folding of the striated muscle myosin MD::GFP is not affected by the addition of Unc45. SDS-PAGE analysis confirms that both MD::GFP chimeras are efficiently synthesized in the assay, but only the smooth muscle MD::GFP chimera is folded.

In *C. elegans*, UNC-45 also acts with non-muscle myosin II (NMY-2) during establishment of embryonic polarity, cytokinesis, and germ line cellularization. Depletion of UNC-45 with RNA interference inhibits contractility of the actomyosin cytoskeleton but does not completely block co-localization of the NMY-2 with actin (20). Non-muscle myosin II is closely related to the smooth muscle myosin. We show here that smooth myosin MD::GFP synthesized in the absence of Unc45a can bind actin, but the interaction is ATP-insensitive, a hallmark of an inactive myosin. Furthermore, the conformation heterogeneity of the protein indicates that not only is it inactive, it is partially unfolded. Therefore, maturation of the actin binding activity of MD::GFP correlates with the formation of the native conformation, and this step is Hsp90-dependent and accelerated by Unc45a.

The levels of UNC-45 in *C. elegans* appear to be tightly controlled by ubiquitinylation and regulated protein degradation (31). It has been suggested that UNC-45 in turn regulates sarcomere assembly through myosin ubiquitinylation and degradation (32). This mechanism is very different from the direct role of Unc45 in myosin folding demonstrated here. Reticulocyte lysate contains the essential ubiquitin-proteasome system necessary for degradation, but we do not see motor domain turnover induced by high concentrations of either vertebrate Unc45 isoform in the lysate even when Hsp90 activity is inhibited (33). So, although myosin levels or turnover also might be regulated by Unc45, a primary activity is that of a co-chaperone in Hsp90-mediated myosin motor domain folding.

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REFERENCES

1. Foth, B. J., Goedecke, M. C., and Soldati, D. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 3681–3686
2. Rayment, I., Rypniewski, W. R., Schmidt-Base, K., Smith, R., Tomchick, D. R., Benning, M. M., Winkelmann, D. A., Wesenberg, G., and Holden, H. M. (1993) *Science* **261,** 50–58
3. Chow, D., Srikakulam, R., Chen, Y., and Winkelmann, D. A. (2002) *J. Biol. Chem.* **277,** 36799–36807
4. Srikakulam, R., and Winkelmann, D. A. (1999) *J. Biol. Chem.* **274,** 27265–27273
5. Srikakulam, R., and Winkelmann, D. A. (2004) *J. Cell Sci.* **117,** 641–652
6. Du, S. J., Li, H., Bian, Y., and Zhong, Y. (2008) *Proc. Natl. Acad. Sci. U. S. A.* **105,** 554–559
7. Hawkins, T. A., Haramis, A. P., Etard, C., Prodromou, C., Vaughan, C. K.,

Ashworth, R., Ray, S., Behra, M., Holder, N., Talbot, W. S., Pearl, L. H., Strahle, U., and Wilson, S. W. (2008) *Development* (*Camb.*) **135,** 1147–1156
8. Murphy, P. J., Kanelakis, K. C., Galigniana, M. D., Morishima, Y., and Pratt, W. B. (2001) *J. Biol. Chem.* **276,** 30092–30098
9. Venolia, L., and Waterston, R. H. (1990) *Genetics* **126,** 345–353
10. Ao, W., and Pilgrim, D. (2000) *J. Cell Biol.* **148,** 375–384
11. Barral, J. M., Bauer, C. C., Ortiz, I., and Epstein, H. F. (1998) *J. Cell Biol.* **143,** 1215–1225
12. Scheufler, C., Brinker, A., Bourenkov, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F. U., and Moarefi, I. (2000) *Cell* **101,** 199–210
13. Barral, J. M., Hutagalung, A. H., Brinker, A., Hartl, F. U., and Epstein, H. F. (2002) *Science* **295,** 669–671
14. Berteaux-Lecellier, V., Zickler, D., Debuchy, R., Panvier-Adoutte, A., Thompson-Coffe, C., and Picard, M. (1998) *EMBO J.* **17,** 1248–1258
15. Wesche, S., Arnold, M., and Jansen, R. P. (2003) *Curr. Biol.* **13,** 715–724
16. Lord, M., and Pollard, T. D. (2004) *J. Cell Biol.* **167,** 315–325
17. Wong, K. C., Naqvi, N. I., Iino, Y., Yamamoto, M., and Balasubramanian, M. K. (2000) *J. Cell Sci.* **113,** 2421–2432
18. Mishra, M., D'Souza, V. M., Chang, K. C., Huang, Y., and Balasubramanian, M. K. (2005) *Eukaryot. Cell* **4,** 567–576
19. Kachur, T., Ao, W., Berger, J., and Pilgrim, D. (2004) *J. Cell Sci.* **117,** 5313–5321
20. Kachur, T. M., Audhya, A., and Pilgrim, D. B. (2008) *Dev. Biol.* **314,** 287–299
21. Price, M. G., Landsverk, M. L., Barral, J. M., and Epstein, H. F. (2002) *J. Cell Sci.* **115,** 4013–4023
22. Dominguez, R., Freyzon, Y., Trybus, K. M., and Cohen, C. (1998) *Cell* **94,** 559–571
23. Winkelmann, D. A., and Lowey, S. (1986) *J. Mol. Biol.* **188,** 595–612
24. Laemmli, U. K. (1970) *Nature* **227,** 680–685
25. Chadli, A., Graham, J. D., Abel, M. G., Jackson, T. A., Gordon, D. F., Wood, W. M., Felts, S. J., Horwitz, K. B., and Toft, D. (2006) *Mol. Cell. Biol.* **26,** 1722–1730
26. Prodromou, C., Roe, S. M., O'Brien, R., Ladbury, J. E., Piper, P. W., and Pearl, L. H. (1997) *Cell* **90,** 65–75
27. Zhao, R., and Houry, W. A. (2007) *Adv. Exp. Med. Biol.* **594,** 27–36
28. Buchner, J. (1999) *Trends Biochem. Sci.* **24,** 136–141
29. Srikakulam, R., Liu, L., and Winkelmann, D. A. (2008) *PLoS One,* in press
30. Wohlgemuth, S. L., Crawford, B. D., and Pilgrim, D. B. (2007) *Dev. Biol.* **303,** 483–492
31. Hoppe, T., Cassata, G., Barral, J. M., Springer, W., Hutagalung, A. H., Epstein, H. F., and Baumeister, R. (2004) *Cell* **118,** 337–349
32. Landsverk, M. L., Li, S., Hutagalung, A. H., Najafov, A., Hoppe, T., Barral, J. M., and Epstein, H. F. (2007) *J. Cell Biol.* **177,** 205–210
33. Hoffman, L., Pratt, G., and Rechsteiner, M. (1992) *J. Biol. Chem.* **267,** 22362–22368