Introduction

Myosins are actin-based motors that play essential roles in a variety of cellular processes, including cytokinesis, cellular trafficking, phagocytosis, maintenance of cell shape, and muscle contraction (Berg et al., 2001). Myosin-based movement results from a precise cycle of the myosin head binding and releasing ATP and actin. During this process, the myosin head transitions through multiple folding conformations.

Molecular chaperones appear necessary for de novo folding and structural maintenance of the myosin head. Expression of the myosin motor domain in bacteria results in misfolding (McNally et al., 1988). In vertebrate systems, the chaperonin containing TCP-1 (CCT), as well as molecular chaperones Hsp90 and Hsc70, are necessary but not sufficient in the folding of striated muscle myosin (Srikakulam and Winkelmann, 1999; Srikakulam and Winkelmann, 2004).

Evidence from a variety of experimental systems indicates that myosins use specialized chaperones during their activity, folding, and assembly. Mutations in UNC-45/Cro1p/She4p(Dim1p) domain (UCS) proteins lead to phenotypes related to defects in myosin folding and assembly (Hutagalung et al., 2002). Decreased UCS domain protein function in fungal mutants produces myosins defective in actin:ATP transduction (Wesche et al., 2003; Lord and Pollard, 2004). In Caenorhabditis elegans, null unc-45 alleles lead to embryonic arrest of body wall muscle development (Venolia and Waterston, 1990), and temperature-sensitive mutations lead to a paralyzed or uncoordinated phenotype at the restrictive temperature with marked disorganization of myofibrils (Epstein and Thomson, 1974; Barral et al., 1998). In vitro, UNC-45 exerts chaperone activity on the myosin head and acts as a cochaperone that specifically binds Hsp90 (Barral et al., 2002).

UNC-45 has recently been shown to be a substrate of an E3/E4-multiquitination complex containing CHN-1 (the C. elegans homologue of CHIP) and UFD-2 (Hoppe et al., 2004). In Caenorhabditis elegans, null unc-45 alleles lead to embryonic arrest of body wall muscle development (Venolia and Waterston, 1990), and temperature-sensitive mutations lead to a paralyzed or uncoordinated phenotype at the restrictive temperature with marked disorganization of myofibrils (Epstein and Thomson, 1974; Barral et al., 1998). In vitro, UNC-45 exerts chaperone activity on the myosin head and acts as a cochaperone that specifically binds Hsp90 (Barral et al., 2002).
shown that unassembled components of myofilaments are degraded through the ubiquitin/proteasome system (UPS) in muscle wasting conditions, including cancer and starvation (Koohmaraie, 1992; Solomon and Goldberg, 1996; Solomon et al., 1998; Acharyya et al., 2004). Our results suggest that enhanced levels of UNC-45 may promote nonnative myosin conformations, rendering them susceptible to degradation by the UPS.

Results and discussion

Wild-type levels of UNC-45 are critical for proper myofilament assembly

Loss-of-function (Lof) temperature-sensitive UNC-45 mutants result in severely paralyzed worms, with pronounced disorganization of the sarcomere (Epstein and Thomson, 1974; Barral et al., 1998). To investigate the consequences of increased UNC-45 levels in a wild-type background, we used the transgenic line, *sul2 [Punc-54::unc-45FLAG]*, expressing UNC-45 under control of the strong muscle–specific *unc-54* promoter. In contrast to lines overexpressing UNC-45FLAG from an extrachromosomal array, this line stably transmits the transgene from generation to generation. *sul2* worms overexpress UNC-45 at ~10-fold greater amounts than that of the previously studied integrated line *sul1 [Punc-54::unc-45FLAG]* (Hoppe et al., 2004; Fig. 1 A).

Although not as pronounced as in Lof alleles, the increased concentrations of UNC-45 in *sul2* also lead to abnormal thick filament assembly and a concomitant defect in movement. We measured body bend rates and found that *sul2* worms were 36% slower than wild type (Fig. 1 B). However, this is not as severe as the temperature-sensitive *unc-45(e286)* mutants grown at the restrictive temperature, which is the result of diminished, rather than augmented, UNC-45 function. Transgenic UNC-45FLAG was able to rescue the motility defects...
of unc-45(e286) at the restrictive temperature, indicating that it is functional (Fig. 1 B).

C. elegans young adult (Ya) wild-type muscle cells are 120–150 μm in length with a mean of seven to nine myosin thick filament–containing A bands per cell (Fig. 1 C, Mackenzie et al., 1978a). Similarly staged suIs2 worms demonstrated cells 108–130 μm in length that contained only five to six A bands (Fig. 1, C and D). Thus, UNC-45 overexpression in suIs2 worms results in a decreased number of myosin-containing thick filaments. This phenotype arises specifically from increased levels of UNC-45FLAG. Transgenic overexpression of the unrelated protein GFP under control of the unc-54 promoter does not result in altered muscle cells (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200607084/DC1). Furthermore, integration of the UNC-45FLAG transgene by itself is not responsible for the muscle phenotype, as the expression of UNC-45FLAG as an extrachromosomal array also decreased the number of myosin-containing A bands in cells in which it was expressed (Fig. S1 B). Moreover, in rare instances in which individual muscle cells in suIs2 worms expressed undetectable levels of UNC-45FLAG, a wild-type phenotype was observed (Fig. S1 C).

Altered UNC-45 levels lead to decreased myosin accumulation

We next investigated whether the overall myosin content was decreased in these worms. Four distinct myosin heavy chain (MHC) isoforms are expressed in C. elegans as major components of the sarcomere (Miller et al., 1983; Dibb et al., 1989). MHC A and B are present in the body wall muscles, whereas MHC C and D are located in the minor pharyngeal muscles (Ardizzi and Epstein, 1987). To assess myosin levels, individual worms were analyzed for MHC A, B, and D. In contrast to MHC D, which was not affected because UNC-45FLAG is specifically expressed in body wall muscle, both body wall MHC A and B were decreased in suIs2 to 70% of wild type (Fig. 2 A). Worms homozygous for the e286 allele grown at the restrictive temperature resulted in decreased accumulation of all myosin isoforms examined (Fig. 2 A). This is expected, as endogenous UNC-45 is involved in pharyngeal as well as body wall muscle myosin assembly (Venolia and Waterston, 1990). Previous results showing that MHC A content was not substantially affected in e286 mutant worms may be explained by the fact that the protein paramyosin was used for normalization. Paramyosin is also a major component of thick filaments, and e286 worms at the restrictive temperature have a 45% mean decrease in number of thick filaments compared with worms at the permissive temperature (Barral et al., 1998). Paramyosin levels are also decreased in suIs2 worms, indicating a loss of total thick filaments (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200607084/DC1).

Because our transgenic line carries multiple copies of the unc-54 promoter, the possibility existed that the observed muscle phenotype was caused by its excessive binding of transcription factors. However, real-time RT-PCR verified that mRNA levels of both myo-3 and unc-54, which encode MHC A and B, respectively, were not significantly different between N2 wild-type and suIs2 worms (Fig. 2 C) with GAPDH mRNA as a control. These results indicated that the decrease in body wall myosin is not a result of diminished transcription.

The UPS is responsible for decreased myosin accumulation in worms overexpressing UNC-45

To examine a role for the UPS in the degradation of endogenous myosin in C. elegans, we first tested whether we could detect ubiquitinated myosin species in whole worm lysates. We performed pull downs from wild-type worms with ubiquitin and antimyosin antibodies and blotted them subsequently with

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Figure 3. The UPS is responsible for degrading myosin in worms overexpressing UNC-45FLAG. (A) C. elegans muscle is ubiquitinated in vivo. Pull-downs from N2 wild-type worms using anti-MHC and antiubiquitin antibodies subsequently subjected to anti-MHC immunoblotting. Bands of slower mobility can be observed in the lane corresponding to the antiubiquitin pull down, which may correspond to multiubiquitinated myosin species. Bands of faster mobility observed in lanes of both pull downs may represent proteolytic fragments of myosin. (B) Degradation of myosin in C. elegans is dependent on ATP and is inhibited by the proteasome inhibitor MG132. Supernatants of worm lysates were incubated with no supplementation, ATP, MG132, or ATP/MG132 at room temperature. Samples were removed at the indicated time points. Western blots to detect MHC B were performed using mAb 28.2. (C) Partial inhibition of proteasomal function through RPT2 RNAi leads to restoration of both MHC isoforms in suIs2 worms. Western blots for MHC A and B were performed on suIs2 worms grown on Escherichia coli containing an empty vector or an RPT2 RNAi–treated worms displayed a mean of 21 and 27 body bends, respectively. *, P < 0.001 versus N2; **, P < 0.001 suIs2 RNAi versus suIs2 empty vector. Error bars indicate SD.
a myosin-specific mAb (Fig. 3 A). The ubiquitin mAb was able to pull down full-length myosin as well as species of slower mobility, consistent with polyubiquitinated myosin species. Several smaller myosin-immunoreactive bands were also detected in both pull downs, which may represent ubiquitinated proteolytic fragments of myosin, consistent with previous studies (Zdinak et al., 1997; Ikemoto et al., 2001; Acharyya et al., 2004; Du et al., 2004). Sarcomeric myosin can thus be ubiquitinated in C. elegans. Elevated ubiquitination would lead to accelerated degradation, which in fact was observed in lysates of suIs2 and unc-45(e286) worms at 25°C when compared with N2 and unc-45(e286) worms at 15°C (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200607084/DC1).

Proteasome-dependent proteolytic degradation requires ATP and is inhibited by MG132 (Rock et al., 1994). We thus tested whether degradation of endogenous C. elegans myosin exhibited these properties. We incubated separate reactions containing crude wild-type worm extracts with no additions, ATP supplementation, MG132 supplementation, and a combination of both, and examined the levels of full-length myosin and its degradation products by immunoblotting (Fig. 3 B). The un-supplemented reaction exhibited constant levels of full-length myosin, whereas the reaction supplemented with ATP showed a clear reduction of full-length myosin over time. MG132 by itself had no effects on full-length myosin levels, but the observed ATP-dependent degradation was effectively blocked by addition of MG132. The proteasome is therefore capable of degrading myosin in C. elegans.

We next tested whether proteasome inhibition could restore the diminished myosin levels observed in the suIs2 worms that overexpress UNC-45 FLAC. RNAi against the proteasomal subunit RPT-2 has been previously used to inhibit proteasome function in living C. elegans worms (Hoppe et al., 2004). Importantly, at the concentration used (see Materials and methods), RPT-2 RNAi did not result in complete inhibition of the proteasome, which allowed the nematodes to survive. When RPT-2 RNAi was fed to suIs2 worms, we observed clear restoration of body wall myosin (Fig. 3, C and D). Furthermore, RPT-2 RNAi also significantly improved the mobility of suIs2 nematodes (Fig. 3 E). Because UNC-45 itself is subject to degradation by the UPS, the previous observation that unc-45 Lof mutants are rescued by RPT-2 RNAi (Hoppe et al., 2004) can be explained by increased levels of UNC-45, myosin, or both. Our current results show that endogenous myosin can be ubiquitinated in C. elegans and that proteasome inhibition can prevent its degradation, in both a wild-type background as well as that resulting from increased UNC-45 function.

Implications for mechanism and muscle disease
We have found that UNC-45 overexpression results in diminished myosin accumulation and assembly because of its increased degradation via the UPS. Lof mutants also show similar but enhanced defects. These results allow us to propose an idealized model in which precise levels of UNC-45 are critical for supporting adequate myosin folding and assembly into thick filaments (Fig. 4). As a myosin chaperone (Barral et al., 2002), UNC-45 binds to newly synthesized myosin motor domains that have not yet attained their full native structure. UNC-45 assists in folding of the motor domain so that it becomes competent for assembly. We hypothesize that myosin produced in the context of UNC-45 Lof mutations is less capable of attaining its native structure and, as a result, is degraded by the UPS, thus explaining its drastically reduced levels (Fig. 2 A) and aberrant assembly into thick filaments (Barral et al., 1998). Myosin synthesized in the context of excess UNC-45 may also be prevented from reaching the assembled state because of mass action and is then susceptible to degradation via the UPS. Because both endogenous and transgenic UNC-45 can associate with assembled myosin in vivo (Ao and Pilgrim, 2000; unpublished data), excess UNC-45 may also be capable of shifting the myosin equilibrium from the assembled state into a degradation-susceptible unassembled state. The interplay between UNC-45, myosin, and the UPS may be of relevance to similar concentration-dependent phenomena between chaperones and client proteins.
capable of polymerization, such as those reported for Hsp104 and yeast prions (Chernoff et al., 1995).

Vertebrates contain two distinct genes encoding different UNC-45 isoforms. General cell UNC-45 is expressed in multiple tissues and appears necessary for various cytoskeletal functions, whereas striated muscle (SM) UNC-45 is specifically expressed in heart and skeletal muscle and may be necessary for sarcomere organization (Price et al., 2002). SM UNC-45 may function during sarcomere assembly in a mechanism similar to the one described here for C. elegans UNC-45. Alterations in human SM UNC-45 function or concentrations may be significant in hypertrophy, dilation, and failure of the heart, as well as in skeletal muscle wasting in a variety of human disorders.

Materials and methods

General C. elegans methods

Nematode strains were grown under standard conditions (Brenner, 1974). suls2 was created as previously described (Hoppe et al., 2004). Genomic integration of the Punc-45::unc-45LAG extrachromosomal array was performed via gamma irradiation (Greenwald and Horvitz, 1980). The resulting worms were outcrossed five times. Strain BC10095 was obtained from D. Baillie (Simon Fraser University, Burnaby, Canada).

Immunoblotting

In Fig. 2 A, Fig. 3 C, and Fig. S2, Ya worms were hand-picked, placed in SDS sample buffer, and heated at 95°C for 10 min. Amounts corresponding to a single worm were loaded per lane. In Fig. S3, equal volumes of L4 Ya stage worms were lysed in 50 mM NaPO4, pH 7.4, 200 mM NaCl, 5 mM MgCl2, 1% Triton X-100, 5 mM DTT, and 5 mM ATP. Samples were resolved by SDS-PAGE and transferred to Hybond nitrocellulose (GE Healthcare). The homogenate was centrifuged at 5,000 g for 10 min at 4°C, and the supernatant was divided into four aliquots to which no supplementation, 2 mM ATP, and/or 100 μM proteasome inhibitor MG132 were added. Samples were kept at room temperature, and aliquots were removed at each time point, mixed with SDS-PAGE sample buffer, and immediately heated at 95°C for 10 min. Samples were resolved by SDS-PAGE followed by Western blots using 5 μg/ml mAb 28.2.

Pull-down assays

N2 worms were lysed and incubated with 100 μg mAb 28.2 or 200 μg anti-ubiquitin mAb (Santa Cruz Biotechnology, Inc.) bound to protein G Sepharose (GE Healthcare) for 2 h at 4°C. Pull downs were washed five times with lysis buffer, mixed with SDS sample buffer, and heated at 95°C for 10 min. Samples were separated by SDS-PAGE followed by Western blotting using 5 μg/ml mAb 28.2. Fivefold less volume of the MHC B pull down compared with the ubiquitin pull down was loaded to avoid saturation.

Online supplemental material

Fig. S1 shows that the suls2 phenotype arises specifically from UNC-45FLAG overexpression. Fig. S2 shows that paramyosin levels are also decreased in suls2 worms. Fig. S3 shows that myosin accumulation is also decreased in whole worm lysates from suls2 and unc-45(e286) worms at the restrictive temperature. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200607084/DC1.

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