***Caenorhabditis elegans* UNC-96 Is a New Component of M-Lines That Interacts with UNC-98 and Paramyosin and Is Required in Adult Muscle for Assembly and/or Maintenance of Thick Filaments**[□]

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To gain further insight into the molecular architecture, assembly, and maintenance of the sarcomere, we have carried out a molecular analysis of the UNC-96 protein in the muscle of *Caenorhabditis elegans*. By polarized light microscopy of body wall muscle, *unc-96* mutants display reduced myofibrillar organization and characteristic birefringent “needles.” By immunofluorescent staining of known myofibril components, *unc-96* mutants show major defects in the organization of M-lines and in the localization of a major thick filament component, paramyosin. In *unc-96* mutants, the birefringent needles, which contain both UNC-98 and paramyosin, can be suppressed by starvation or by exposure to reduced temperature. UNC-96 is a novel ~47-kDa polypeptide that has no recognizable domains. Antibodies generated to UNC-96 localize the protein to the M-line, a region of the sarcomere in which thick filaments are cross-linked. By genetic and biochemical criteria, UNC-96 interacts with UNC-98, a previously described component of M-lines, and paramyosin. Additionally, UNC-96 copurifies with native thick filaments. A model is presented in which UNC-96 is required in adult muscle to promote thick filament assembly and/or maintenance.

INTRODUCTION

The myofibril is a complex assemblage of many proteins, with new components being discovered each year. Despite this growing understanding about myofibrillar components, their interactions, and individual functions, we still know little about the assembly of this precise structure (Gregorio and Antin, 2000). Additionally, it is unclear how this structure is maintained in the face of repeated contraction and relaxation. The need to understand the mechanisms in charge of myofibrillar maintenance is underscored by the clinical significance of degenerative muscular atrophies and myopathies. *Caenorhabditis elegans* is a particularly attractive organism in which to address these problems (Waterston, 1988; Moerman and Fire, 1997; Moerman and Williams, 2006). We are able to view the muscle in a whole animal system, allowing us to focus on the true, extracellular matrix (ECM) attachment properties of the muscle cell, an evaluation not possible with tissue culture. Because of the optical transparency of the worm, we are able to visualize the

myofibrillar structure by polarized light and localize green fluorescent protein (GFP)-tagged proteins in live worms. A major strength of *C. elegans* lies in its ability to grow quickly and reproduce in large numbers, allowing genetic analyses in a timely and efficient manner. Last, its usual self-fertilization permits propagation of muscle mutants that would otherwise be unable to mate.

In the nematode, most of the muscle is located in the body wall and is used for locomotion. Throughout the muscle cell, the thin filament attachment structures, called dense bodies (analogous to the Z-discs in vertebrate muscle) and the thick filament cross-linking structures, the M-lines, are anchored to the muscle cell membrane. This permits the force of contraction to be transmitted through the cell membrane, the basement membrane, and hypodermis to the overlying cuticle, resulting in movement of the whole animal. Thus, in addition to their roles in attaching thin filaments and cross-linking thick filaments, nematode dense bodies and M-lines are analogous to vertebrate focal adhesion plaques because of their membrane anchorage and composition.

Studies during the past 30 years in over a dozen laboratories have defined many components of *C. elegans* myofibrils and their membrane–ECM attachment structures. Most of these proteins were first defined through mutations, most falling into one of two phenotypic classes. The first class, the uncoordinated or “Unc” class, has slow moving or paralyzed adults. The second class of mutants, paralyzed arrested at two-fold (“Pat”), has a characteristic embryonic lethality in which embryos do not move within the eggshell and stop development at the twofold stage (Williams and Waterston, 1994). A model for myofibril assembly has been

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Abbreviations used: GFP, green fluorescent protein; GST, glutathione S-transferase; MHC A, myosin heavy chain A; MHC B, myosin heavy chain B; MBP, maltose-binding protein; SEs, standard errors.

proposed (Williams and Waterston, 1994; Moerman and Williams, 2005) in which assembly is initiated or directed by signals first laid down in the ECM and muscle cell membrane. This is supported by the observation that the Pat mutants showing the greatest degree of disorganization define genes that encode components of the ECM (e.g., UNC-52 [perlecan]) and muscle cell membrane (e.g., PAT-3 [β -integrin] and PAT-2 [α -integrin]). This model is further supported by immunolocalization studies revealing when and where these proteins are localized in embryonic muscles (Hresko *et al.*, 1994). It has become clear that proteins at the base of dense bodies, that is, at or near the cell membrane, are the same as proteins found at the base of M-lines. However, protein components, specific to each structure, reside more internally (further away from the cell membrane). Thus, in the extracellular matrix, concentrated beneath both dense bodies and M-lines is the nematode homologue of perlecan, UNC-52 (Rogalski *et al.*, 1993). Within the muscle cell membrane, localized at the bases of both dense bodies and M-lines, are the integrins, including PAT-3- β -integrin (Williams and Waterston, 1994; Gettner *et al.*, 1995) and PAT-2- α -integrin (Williams, personal communication). Traveling further internally, both dense bodies and M-lines contain talin (Moulder *et al.*, 1996), UNC-97 (mammalian PINCH; Hobert *et al.*, 1999), UNC-112 (Mig-2; Rogalski *et al.*, 2000), PAT-4 (integrin-linked kinase; Mackinnon *et al.*, 2002), PAT-6 (actopaxin; Lin *et al.*, 2003), UNC-98 (Mercer *et al.*, 2003), and UNC-95 (Broday *et al.*, 2004). Vinculin (DEB-1; Barstead and Waterston, 1991) and α -actinin (Francis and Waterston, 1985) are found specifically in the dense bodies, whereas UNC-89 is found only in the M-lines (Small *et al.*, 2004). UNC-98 seems to be enriched at the M-lines. Although UNC-98::GFP can be found at both M-lines and dense bodies, anti-UNC-98 antibodies only label the M-lines, unless UNC-98 is overexpressed (Mercer *et al.*, 2003). Most mutants in *unc-89* or *unc-98* show either a lack of M-lines or short or broken M-lines (Benian *et al.*, 1996; Mercer *et al.*, 2003).

In *C. elegans* adult body wall muscle, thick filaments are $\sim 10\ \mu\text{m}$ in length and are organized around an M-line (Waterston, 1988). The three major components of these thick filaments are myosin heavy chain A (MHC A), myosin heavy chain B (MHC B) and paramyosin, encoded by the genes *myo-3*, *unc-54*, and *unc-15*, respectively (Epstein *et al.*, 1974; Miller *et al.*, 1986; Kagawa *et al.*, 1989). Homodimers of the two myosin heavy chains (Schachat *et al.*, 1978) are differentially localized: MHC A in the center and MHC B in the polar regions (Miller *et al.*, 1983). Paramyosin is primarily an α -helical coiled-coil rod and is 38% identical in amino acid sequence to the rod domains of myosin heavy chains. The myosins and a subpopulation of paramyosin are organized around a tubular core (Deitiker and Epstein, 1993). The cores are composed of a distinct subpopulation of paramyosin together with the α , β , and γ -filagenins, in a specific geometry (Epstein *et al.*, 1995; Muller *et al.*, 2001). Because *myo-3* null mutants do not form thick filaments and are Pat embryonic lethal (Waterston, 1989), MHC A is required for either the initiation or stabilization of thick filament assembly. *unc-15* mutants lacking paramyosin have myosin aggregates and form very thin, abnormal filaments, resulting in a severely paralyzed adult animal (Waterston *et al.*, 1977). MHC B is nonessential for thick filament assembly, because *unc-54* (MHC B) null mutants can be suppressed by twofold overexpression of MHC A (Riddle and Brenner, 1978; Maruyama *et al.*, 1989). During development, the differential expression of the different filagenin genes seems to be important for the assembly of thick filaments of distinct

lengths (Liu *et al.*, 2000). Other components of nematode thick filaments likely include twitchin and UNC-45. Twitchin is a 754,000-Da polypeptide related to mammalian titin and is encoded by the *unc-22* gene (Benian *et al.*, 1989, 1993). *unc-22* mutants have a characteristic "twitching" phenotype and variably disorganized muscle structure in which thick filaments are present but not organized into A-bands (Waterston *et al.*, 1980; Moerman *et al.*, 1988). Twitchin is localized to the outer portions of A-bands, colocalizing with MHC B (Moerman *et al.*, 1988). Although null mutations of *unc-45* result in Pat embryonic lethality, temperature-sensitive missense mutations are *Unc* adults and show decreased accumulation of thick filaments (Barral *et al.*, 1998). Moreover, in these *unc-45* temperature-sensitive mutants, the differential localization of MHC A and B is lost. Biochemically, UNC-45 is a conserved protein that interacts with both Hsp90 and myosin head domains and acts as a chaperone for myosin assembly into thick filaments (Barral *et al.*, 2002). For UNC-45 to function normally, its level has to be tightly regulated by E3/E4 multiubiquitylation (Hoppe *et al.*, 2004).

Here, we report the molecular genetic analysis of UNC-96, a protein required for maintenance of myofibril structure even after myofibrils have been assembled. Specifically, we have found that UNC-96 localizes to the M-line where it is likely to play an important role in A-band integrity. This result is supported by data revealing that UNC-96 interacts with UNC-98 (another M-line component) and paramyosin (a thick filament component) and is present in a preparation of purified thick filaments. We present a model in which UNC-96 is required in adult muscle and acts as both an M-line structural component and as a facilitator of thick filament assembly and/or turnover, through its association with unincorporated paramyosin and UNC-98.

MATERIALS AND METHODS

Strains and Genetics

There are three mutant alleles of *unc-96*: 1) The original allele *su151* was described by Zengel and Epstein (1980) and was recovered by a motility selection. When we first obtained *unc-96(su151)* from the *Caenorhabditis* Genetics Center, we noted that these worms were obviously slow moving by casual observation. However, after outcrossing twice to wild type, the polarized light defect was retained, but the motility defect was not. Presumably, the original *su151* strain had an additional mutation, which caused the slow motility. 2) *r291* was isolated by P. Anderson as a suppressor of *unc-105* and was kindly sent to us as a presumed allele of *unc-89*. However, by examining its muscle by polarized light and by complementation testing, we found that *r291* is an allele of *unc-96*. 3) In a polarized light F1 noncomplementation screen using deficiency *meDf6*, we recovered *sf18* by ethyl methanesulfonate (EMS) mutagenesis. All three *unc-96* mutant alleles were outcrossed three or more times to wild type before our analysis. To create the trans-heterozygotes between *su151* and *sf18*, or *r291* and *sf18*, we used the *sf18* marked in *cis*-with *unc-1(e538)*. *unc-96(sf18) unc-1(e538)* animals are easily identified by the "kinker" phenotype of *unc-1*. Some experiments used *unc-98(sf19)* (Mercer *et al.*, 2003). *unc-98(RNAi)* animals and *unc-98::gfp* translational fusion transgenic animals were also used (described in Mercer *et al.*, 2003). RNA interference (RNAi) by feeding for predicted genes in cosmid F13C5 (see below) was performed in the RNAi-hypersensitive strain *rrf-3(pk1426)* (Simmer *et al.*, 2002). To generate trans-heterozygotes and double homozygotes between *unc-15* and *unc-96*, we used the following scheme. To follow the *unc-96(sf18)* phenotype with a dissecting microscope, we used *unc-96(sf18) unc-1(e538)*, as described above. We confirmed that *unc-1* has no effect on the muscle phenotype of *unc-15(e1215)*: *unc-15(e1215)*; *unc-1(e538)* doubles move slowly like *unc-15(e1215)* and kink like *unc-1(e538)*. Trans-heterozygous *unc-15(e1215)/+;* *unc-96(sf18) unc-1(e538)/+* animals were generated by crossing *unc-15(e1215)/+* males with *unc-96(sf18) unc-1(e538)* homozygous hermaphrodites. One-half of the resulting non-*unc-1* progeny are trans-heterozygotes. The *unc-1* progeny from the same cross were allowed to self, producing one-quarter *unc-15(e1215)*; *unc-96(sf18) unc-1(e538)* homozygotes.

Polarized Light Microscopy, Motility Assays, and Electron Microscopy

These were performed as described in Mercer *et al.* (2003). Polarized light images were obtained with a Zeiss Axioskop microscope (Carl Zeiss, Jena,

Germany) on Kodak BW400CN print film, scanned, and processed with Adobe Photoshop (Adobe Systems, Mountain View, CA).

Characterization of the Unc-96 Mutant Phenotype by Using Antibodies to Known Myofibril Components

For most experiments, we used the procedure described in Mercer *et al.* (2003). The following antibodies were used: monoclonal antibodies (mAbs) (Miller *et al.*, 1983) to myosin heavy chain A (5-6), myosin heavy chain B (5-8), and paramyosin (5-23); a second mAb to paramyosin, Mab 5B5 (Gengyo-Ando and Kagawa, 1991); affinity-purified rat antibodies to UNC-89 (EU133; Small *et al.*, 2004); and affinity-purified rabbit antibodies to UNC-98 (EU131; Mercer *et al.*, 2003). In the anti-UNC-96 staining of *unc-98(sf19)*, we used the immunostaining method of Nonet *et al.* (1993). Thin filaments were visualized by tetramethylrhodamine-phalloidin staining as described in Ono (2001). Nearly all images were obtained with a Zeiss Axioskop microscope using Fuji Sensia 100 slide film and scanned and processed with Adobe Photoshop. For determining the dependence of UNC-96 localization on the presence of paramyosin, we costained either wild-type or *unc-15(e1214)* null animals with anti-UNC-96 (EU148 at 1:100) and anti- α -actinin (MH35 at 1:200; Francis and Waterston, 1985). Images depicting dual antibody localization were captured with a scientific-grade cooled charge-coupled device (Cool-Snap HQ with ORCA-ER chip) on a multiwavelength, wide-field, three-dimensional microscopy system (Intelligent Imaging Innovations, Denver, CO). Samples were imaged in successive 0.2- μ m focal planes, and out-of-focus light was removed using the constrained iterative deconvolution algorithm (Weiner *et al.*, 1999). Images were processed using Adobe Photoshop software.

Method of Starvation

Escherichia coli strain OP50 seeded NGM plates, containing nearly starved *sf18* and *r291* adult animals, were rinsed three times with M9 buffer. Worms were then transferred to an unseeded NGM plate for 24 or more hours, before being viewed by polarized light. For the motility assays of starved *sf18* and N2, similarly staged animals (young adults) were picked to a small unseeded NGM plate, rinsed three times in M9, and transferred to new unseeded NGM plate. A chunk of NGM plus OP50 was placed on the lid of the Petri dish to prevent the worms from crawling off the plate or under the agar.

Genetic Mapping and Molecular Cloning of *unc-96*

By three-factor mapping with *unc-1(e1598n1201)* and *dpy-3(e27)*, we were able to place *unc-96* to the left of *unc-1*. Although we were never able to definitively determine the left/right positioning of *unc-96* relative to *egl-17*, recombinant data suggested that *unc-96* resides in a cosmid close to F38G1 (containing *egl-17*). We used single nucleotide polymorphism (SNP) mapping (Hill *et al.*, 2000) to narrow down a region of more than 20 overlapping cosmid clones lying to the right of F38G1. Mating of the *C. elegans* Hawaiian strain to an *unc-96 unc-1 dpy-3* triple resulted in a number of non-UNC-96, non-UNC-1 Dpy-3 recombinants. Sequencing of confirmed SNPs lying to the left of *dpy-3*, revealed that *unc-96* lies within or to the left of cosmid F02G3. We then tested several cosmids lying to the left of F02G3 for their ability to rescue the UNC-96 phenotype in transgenic animals. (Cosmid DNAs were prepared using a QIAGEN Plasmid Maxiprep kit; QIAGEN, Valencia, CA). Cosmid F13C5 gave transgenic rescue. RNAi was then performed for all six predicted genes in F13C5 using clones from the Ahringer library (available from GeneService, Cambridge, United Kingdom) and a feeding procedure essentially as described in Kamath and Ahringer (2003). Worms fed bacteria producing double-stranded RNA (dsRNA) for F13C5.6 resulted in the UNC-96 polarized light phenotype.

Sequencing of *unc-96* Mutants and cDNAs, and Production of Recombinant UNC-96 Protein

To determine the mutation sites, we first prepared genomic DNA from each of the three mutant alleles (by phenol/chloroform extraction). We designed primers to amplify genomic sequence of less than or equal to 1 kb that included exonic sequences and ~50–100 base pairs of flanking intronic sequences. In most cases, the primers used to amplify were also used as sequencing primers. All mutation sites were confirmed by a second, opposite direction read.

Forward primer ACTGGTCGACTGATGAGTGAACTGAAGATGTG and reverse primer TTATGCGGCCGCGTCATCTAATGGGTCTTCATA were used to amplify full-length *unc-96* cDNAs (using Triple Master; Eppendorf, Westbury, NY), beginning with a cDNA pool as template. The resulting PCR products were digested with restriction enzymes SalI and NotI and ligated into pET24a (Novagen/EMD Biosciences, San Diego, CA). Multiple clones were sequenced, verifying the presence of two isoforms and cDNA sequence as predicted by WormBase. An error-free clone containing cDNA sequence from the shorter isoform (WormBase, F13C5.6a) was transformed into BL21 codon plus (DE3) RIL competent cells (Stratagene, La Jolla, CA). Expression and purification of His-tagged UNC-96 was accomplished by using the His bind column system (Novagen/EMD Biosciences).

Generation of Transgenic Lines Carrying *unc-96::gfp*

To obtain full-length, genomic sequence for *unc-96*, two PCR products were created and ligated within intronic sequence, resulting in a final, 11.8-kb fragment of DNA. Product 1 containing ~2 kb of presumed promoter sequence upstream of the initiator methionine and the 5' half of *unc-96* coding sequence (5.8 kb) was created using forward primer ATCAGTCGACTTG-GAGCCTTATGAAGCTAAATG and reverse primer TAATGCGGCCGCT-TATCTTAGTTGGTAGTGGTCAG. Product 2 containing the 3' half of *unc-96* (6 kb), including the last codon before the *unc-96* stop codon, was created using forward primer ATTAGCGGCCGCAACAATGTTGCCAACTATAGTG and reverse primer TGATGGATCCGTCATCTAATGGGTCTTCATAATC. The PCR products were digested with restriction enzyme pairs SalI/NotI and NotI/BamHI, respectively. Both fragments were placed together in a ligation reaction with promotorless vector pPD95.77 (kindly provided by Andy Fire, Stanford University, Stanford, CA), and transformed into *E. coli* strain XL1 Blue. Plasmid clones were sequenced at their ends to verify proper left/right positioning of the insert within the vector. The resulting construct is expected to express, using the normal *unc-96* promoter, a full-length UNC-96 protein, with GFP fused at its C terminus. A single clone was injected (10–40 ng/ μ l) along with *rol-6* (80 ng/ μ l), resulting in three transgenic lines. The same injection mix was injected into *unc-96(sf18)*, and the resulting transgenic lines were rescued for the UNC-96 phenotype. An independent construct was also able to rescue *unc-96*. Images of GFP fluorescence in adult body wall and pharyngeal muscle were obtained with a Zeiss Axioskop microscope on Fuji Sensia 100 slide film, scanned, and processed using Adobe Photoshop. Polarized light images of the rescued animals were obtained as described above.

Analysis of UNC-96 Protein Sequence

The full-length protein sequence for both forms of UNC-96 (418 and 408 aa) were analyzed using programs for domain prediction and sequence similarities available at the following Web sites: 1) Pfam, <http://www.sanger.ac.uk/Software/Pfam/>; 2) BLAST, <http://www.ncbi.nlm.nih.gov/BLAST/> using blastp and search for short, nearly exact matches; and 3) Motif Scan http://myhits.isb-sib.ch/cgi-bin/motif_scan. The amino acid composition of UNC-96 was compared with the "average" amino acid composition of the entire *C. elegans* proteome as calculated by Ter-Hovhannisyan and Borodovsky (personal communication).

Generation of anti-UNC-96 antibodies, Western Blots, and Immunofluorescence Microscopy

Polyacrylamide gel slices containing the 6His-tagged UNC-96 protein were supplied to Spring Valley Laboratories (Woodbine, MD) for generation of rabbit antibodies (EU148). Resulting antibodies were affinity purified using an Affigel (Bio-Rad, Hercules, CA) matrix to which the His-tagged UNC-96 protein had been covalently coupled. Extracts of Laemmli-soluble proteins from wild type and the three *unc-96* mutant alleles were prepared by the method of Hannak *et al.* (2002). The protein concentrations of these extracts were determined by the filter paper dye-binding method of Minamide and Bamburg (1990). A Western blot was reacted with affinity-purified anti-UNC-96 at a 1:200 dilution and visualized by enhanced chemiluminescence (ECL) (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). These same antibodies were used to localize UNC-96 in both embryonic and adult muscle. For localization in embryos, we used the method described in Soto *et al.* (2002) with the following modifications: after methanol/acetone fixation, the embryos were gradually rehydrated through a series of solutions of decreasing acetone concentration, and phosphate-buffered saline was substituted for Tris buffer throughout the procedure. Anti-UNC-96 was used at 1:100 dilution and costained with anti-MHC A 5-6 at 1:400 dilution. Images were captured with a Bio-Rad Radiance model 2100 confocal microscopy system and displayed using LaserSharp2000 software. For localization in adult muscles, we used the method described in Mercer *et al.* (2003). Anti-UNC-96 was used at 1:100 dilution and costained with monoclonal antibodies (MH42; Benian *et al.*, 1996) to UNC-89 at 1:200 dilution. Images were acquired with a scientific-grade cooled charge-coupled device (Cool-Snap HQ with ORCA-ER chip) on a multiwavelength, wide-field, three-dimensional microscopy system (Intelligent Imaging Innovation).

Detection of UNC-96 within Thick Filaments

Thick filament preparations from wild-type strain N2 animals were performed as described previously (Epstein *et al.*, 1988; Deitiker and Epstein, 1993). Proteins isolated at each step of the thick filament purification (Figure 12A) were separated on a 4–15% gradient SDS-PAGE gel and transferred to nitrocellulose membrane. The immunoblot was exposed to anti-UNC-96 antibodies (affinity-purified EU148) at a dilution of 1:100. The reaction was detected by ECL Advanced (GE Healthcare). The supernatant from the 5000 $\times$ g spin of the thick filament preparation was loaded on an 18-ml 19–38% sucrose gradient in a 1 $\times$ 3.5-in., 38.5-ml Beckman polyallomer centrifuge tube. The gradient was centrifuged at 6150 $\times$ g in a SW28 rotor for 16 h 20 min at 4°C. Fractions were collected from the bottom of the gradient. Sucrose gradient fractions were pooled as follows: S1-5, S6-10, and S11-14. These pooled fractions were dialyzed against 10 mM NaPO₄, pH

63.6. One volume of ice-cold 95% ethanol was added to each fraction pool. Precipitated proteins were pelleted by centrifuging at $12,000 \times g$ at 4°C for 20 min. The pellets were air-dried at room temperature and suspended in 200 μ l of 2 $\times$ Laemmli sample buffer. The proteins of the fractions were then separated on two 4–15% SDS-PAGE gels. One gel was Coomassie stained. The other was immunoblotted. UNC-96 was detected with anti-UNC-96 antibodies as described above.

Two-Hybrid Experiments

The UNC-96 cDNA fragments were cloned into pGBDU vectors (James *et al.*, 1996), resulting in plasmids expressing UNC-96 fused to the DNA binding domain of Gal4 (bait). The UNC-98 cDNA fragments (described in Mercer *et al.*, 2003) were cloned into pGAD vectors (James *et al.*, 1996), resulting in plasmids expressing UNC-98 fused to activator domain of Gal4 (prey). The UNC-96 bait and UNC-98 prey plasmids were transformed into PJ69-4A yeast cells (James *et al.*, 1996) using the lithium acetate method (Ito *et al.*, 1983). Yeast two-hybrid assays were performed as described in Mackinnon *et al.* (2002).

The UNC-96 (1–200 aa) and UNC-96 (201–418 aa) cDNA fragments were amplified by using the following combination of primers: U96-1 (CGCGCCCGGGATGAGTGAACTGAAGATGTG) and U96-4 (GCGCGGTCGACTTAAGAACCAGCATTAACATATT) for 1–200 aa and U96-6 (CGCGCCCGGGGTCCATATGCAGCGCCTGCA) and U96-2 (GCGCGTCTGACTTAGTCATCTAATGGGTCTTC) for 201–418 aa. Error-free cDNAs were cloned into pGBDU-C1 using SmaI and SalI sites, resulting in pGBDU96-14 and pGBDU96-62, respectively. To clone UNC-96 (45–418 aa) cDNA fragments, the XhoI fragment of H11-10 (Tsuboi *et al.*, 2002) was cloned into pGBDU-C2.

Far Western

A far Western was conducted as follows. To prepare a maltose binding protein (MBP) fusion protein for the C-terminal half of UNC-96, the clone insert from pGBDU96-62 (described above) was excised and cloned into pMAL-KK1 vector (kindly provided by Dr. K. Kaibuchi, Nagoya University, Nagoya, Japan) (resulting protein named 96MBP). pMAL-KK1 with no insert was used to make MBP alone. Full-length UNC-98 with a 6His tag (98His) was made using full-length *unc-98* cDNA cloned into pET-24a (Novagen); construction details to be described elsewhere. The clones were transformed into BL21 codon plus (DE3) RIL cells (Stratagene), and protein expression was induced with isopropyl β -D-thiogalactoside (1 mM final). The proteins were isolated by cell lysis (described above). Both MBP and 96MBP were dialyzed overnight in 20 mM Tris, pH 7.4, and 100 μ M ZnSO₄. ZnSO₄ was found to reduce the degradation of 96MBP. 98His was dialyzed overnight in 50 mM Tris, pH 7.5. Then, 5 μ g of 98His was run per lane on a 10% acrylamide SDS gel and transferred to nitrocellulose. Strips containing the 98His were blocked in milk Tris-buffered saline (TBS)-T for 1 h and then soaked with either MBP (5 μ g/ml in milk TBS-T) or 96MBP (5 μ g/ml in milk TBS-T) overnight at 4°C. After rinsing in TBS-T, the blots were incubated for 40 min with horseradish peroxidase (HRP)-conjugated anti-MBP (1:5000; New England Biolabs, Beverly, MA), and the reactions were visualized by ECL (GE Healthcare). 2.5 μ g of 98His, MBP, and 96MBP were run on a 10% acrylamide and stained with Coomassie.

Enzyme-linked Immunosorbent Assay (ELISA) Showing Binding between Paramyosin and UNC-96

Paramyosin was purified from a population of wild-type worms of mixed developmental stages using the method of Waterston *et al.* (1974). His-tagged UNC-96 was prepared as described above. The Bio-Rad Bradford-based protein assay was used to determine the concentrations of His-tagged UNC-96 and paramyosin. The following steps were followed in performing an ELISA: 1) Paramyosin was coated on Corning polystyrene microtiter plates (catalog no. 3591; Corning Life Sciences, Acton, MA) at a concentration of 0.5 μ M, at 100 μ l per well in 10 mM NaPO₄, pH 7.6, 0.6 M NaCl, and incubated at 4°C overnight. 2) Wells were coated with blocking buffer (0.2% bovine serum albumin [BSA], 100 mM KCl, 10 mM Tris, pH 8.0, and 0.05% Tween 20) for 1.5 h at room temperature. 3) Wells were washed three times with wash buffer (the same as blocking buffer, without BSA) and vacuum aspirated. 4) One hundred microliters of His-tagged UNC-96 protein was added to the wells at concentrations between 0 and 0.5 μ M and incubated for 1 h at room temperature. 5) The washing procedure was repeated. 6) The wells were coated with 75 μ l of anti-6His antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:200 dilution in blocking buffer for 45 min at 37°C. 7) The washing procedure was repeated. 8) The wells were incubated with 50 μ l of donkey anti-rabbit-HRP antibody (GE Healthcare) at a 1:1000 dilution for 45 min at 37°C. (9) The washing procedure was repeated. 10) Wells were coated with 100 μ l of mixed TMB solution (BD Biosciences, San Jose, CA), and the plate was incubated in the dark at room temperature for 20 min. 11) The absorbances were recorded at 650 nm using a Synergy (Reading, PA) HT multi-detection microplate reader with KC4 data analysis software. A control for nonspecific binding of UNC-96 to BSA was performed. The best fit ligand binding curves were determined by plotting means and standard deviations of absorbance values (Sigma Plot 9.0; SPSS, Chicago, IL).

RESULTS

The *unc-96* Mutant Phenotype

Because of the optical transparency and anatomical simplicity of *C. elegans*, polarized light microscopy is a convenient means of assessing the organization of the myofilament lattice of body wall muscle cells. As shown in Figure 1A, polarized light reveals wild-type muscle to be a highly organized structure in which bright A-bands alternate with dark I-bands. As first reported in Zengel and Epstein (1980), *unc-96(su151)* has a much less regular pattern of A- and I-bands and “discrete birefringent needles near the ends of the body wall muscle cells.” To gain further understanding about the *unc-96* mutant phenotype, we identified two additional *unc-96* mutant alleles, *r291* and *sf18*. *r291* was isolated as a suppressor of *unc-105* by Anderson (personal communication), and by complementation testing we found that *r291* is an allele of *unc-96*. We recovered *sf18* after EMS mutagenesis by a polarized light F1 noncomplementation screen using a deficiency (*meDf6*). Both *unc-96(r291)* and *unc-96(sf18)* have a polarized light appearance identical to that of the original allele, *su151* (Figure 1A).

To understand the role of *unc-96* in muscle function, we compared the motility of *unc-96* mutants to that of wild type. By use of a quantitative measure of the ability of the worms to swim in liquid (Figure 1B), *unc-96(r291)* and *unc-96(su151)* are nearly as fast as wild type. *sf18* is the slowest of the *unc-96* mutant alleles and significantly slower than wild type. Because *unc-96(sf18)* in trans to a noncomplementing deficiency (*meDf6*) is equally slow to an *unc-96(sf18)* homozygote, it is likely that the *unc-96* mutation in *sf18* results in this motility defect. However, given that *sf18* is the only allele that shows a motility defect and that neither *su151* nor *r291* worsen in trans to *sf18*, we cannot rule out the possibility that the motility defect is due to mutations closely linked to *unc-96* in *sf18*.

To further characterize the structural defect in adult *unc-96* mutant muscle, we used immunofluorescent microscopy to localize a number of known myofibril components. We obtained similar results with all three mutant alleles. As shown in Figure 2, *unc-96* mutants display only slight abnormalities in the organization of myosin heavy chains A and B (MHC A and MHC B) and F-actin, but they display significant abnormalities in the organization of UNC-89 and paramyosin. UNC-89 is actually a set of six different polypeptides all localized to the M-line region (Small *et al.*, 2004; Ferrara *et al.*, 2005). Paramyosin is an invertebrate-specific coiled-coil rod protein, similar to the rod portion of myosin heavy chains, and is located in the center of thick filaments (Epstein *et al.*, 1985; Kagawa *et al.*, 1989). Instead of the regular, continuous and linear localization in wild type, in *unc-96*, UNC-89 localizes to noticeably shorter, discontinuous lines. In *unc-96* mutants, paramyosin not only localizes normally to A-bands but also localizes abnormally as accumulations at the ends of the muscle cells. Presumably, these accumulations correspond to the birefringent needles observed by polarized light. Identical anti-paramyosin localization was found with two, independently generated monoclonal antibodies, 5-23 (Miller *et al.*, 1983) and Mab 5B5 (Gengyo-Ando and Kagawa, 1991); in Figure 2, results of 5-23 staining are shown. Immunostaining of *unc-96* mutants for vinculin and α -actinin, major components of dense bodies, also reveals a somewhat abnormal staining pattern with α -actinin being more strongly affected (our unpublished data). Thus, the *unc-96* phenotype involves major disruptions of M-lines and the localization of paramyosin and also minor abnormalities in dense bodies, I-bands, and A-bands.

Figure 1. *unc-96* mutants are defective in muscle structure and motility. (A) Polarized light microscopy of adult body wall muscle. In muscle from wild type, note the regular striated pattern of bright A-bands alternating with dark I-bands. In muscle from the three *unc-96* mutants and the F13C5.6 (RNAi) animals, note the reduced level of organization and appearance of birefringent needles at the ends of the muscle cells. The Unc-96 phenotype can be seen in the young adult worms (P₀) fed F13C5.6 (*unc-96*) dsRNA for 48 h, indicating a requirement for new UNC-96 synthesis in adult worms. Rescue of the Unc-96 phenotype is achieved using an extrachromosomal array containing the putative promoter sequence and complete coding sequence of F13C5.6 (*unc-96*) translationally fused to

Figure 2. Immunofluorescent localization of myofibril components in wild-type and *unc-96* mutant muscle. Antibodies for thick filament proteins (MHC A, MHC B, and paramyosin), an M-line protein (UNC-89), or phalloidin for staining the F-actin in thin filaments were used. *unc-96* mutants show minor disruptions in the organization of MHC A, MHC B, and F-actin but severe disorganization of UNC-89. *unc-96* mutants also display an abnormal staining pattern with antibodies to paramyosin: although some paramyosin is normally localized to A-bands, much paramyosin is found in accumulations located at the ends of the muscle cells. Bar, 10 μm.

Myofibrillar Structure but Not Motility of *unc-96* Mutants Is Suppressed by Lower Temperature

Each mutant strain was grown, beginning as embryos, at three temperatures: 20°C, the standard growth temperature for *C. elegans* in the laboratory; lower temperature, 15°C; and elevated temperature, 25°C. Assessment of adult muscle structure by polarized light microscopy revealed no obvious differences between animals grown at 20 and 25°C (our unpublished data). However, for all three *unc-96* mutant alleles, the muscle structural defects were partially sup-

GFP. Bar, 20 μm. (B) Motility assays on adult worms of the indicated genotypes. Data are shown as means and SEs with *n* = 25–50.

Figure 3. Myofibrillar structure but not motility of *unc-96* mutants is suppressed by lower temperature, and both myofibrillar structure and motility of *unc-96* mutants are suppressed by starvation. (A and B) Polarized light microscopy of body wall muscle of adult *unc-96* mutants of the indicated genotypes at the indicated growth conditions. Notice that at the standard growth temperature for *C. elegans* in the laboratory, 20°C, the typical phenotype of reduced myofibrillar organization and birefringent needles is evident. However, when grown from the embryonic stage at the reduced temperature, 15°C, there are fewer needles, and those that are present, are thinner. When *r291* was grown at this temperature, there is almost a complete loss of needles. This suppression of the needle phenotype is found even when the shift to lower temperature (for 24 h) is delayed until the young adult (YA) stage. Greater improvement in muscle structure is obtained if young adults are deprived of *E. coli* food (starve) for ~24 h (at 20°C). Similar results from reduced temperature or starvation were obtained with the third allele, *su151* (our unpublished data). (C) Motility assays on adult worms of *unc-96(sf18)* and wild type (wt) under the indicated growth conditions (standard, 20°C; growth at 15°C from the embryonic stage; food deprivation for 24 h beginning as young adults). Note that lower temperature does not improve the motility of *sf18* animals to wild-type levels. Significantly, starvation improves the motility of both wild-type and *sf18* animals. Numbers are expressed as means with SEs and $n = 20-50$ for all genotypes and experiments. (D) Colabeling of anti-UNC-89 and anti-paramyosin antibodies in body wall muscle of a young adult *unc-96(sf18)* that had been deprived of food (starve) for the prior 24 h. Note the greatly improved muscle structure, including more organized M-lines (UNC-89) and the absence of abnormally localized paramyosin in distinct accumulations. (E) Polarized light microscopy demonstrating that previously starved *unc-96(sf18)* animals show restoration of the Unc-96 needle phenotype upon reexposure to food.

Figure 4. Molecular cloning of *unc-96* and the sequence alterations in three *unc-96* mutants. Genetic and physical map of the *unc-96* region on the left arm of linkage group X. Three factor mapping placed *unc-96* to the left of *unc-1*. SNP mapping placed *unc-96* further to the left, within or to the left of cosmid F02G3. DNA from cosmids lying to the left of F02G3 and right of *egl-17* were injected into *unc-96(sf18)* animals, and this resulted in identifying a single cosmid, F13C5, capable of rescuing the *Unc-96* mutant phenotype. The six predicted genes in this cosmid sequence were tested by feeding RNAi. F13C5.6 (RNAi) gave a polarized light phenotype similar to that of *unc-96(sf18)*. Sequencing of the three alleles revealed that *r291* and *su151* have GT to AT changes that disrupt normal splice donor sites and that *sf18* has a nonsense codon in exon 9.

pressed at 15°C. As shown in Figure 3, A and B, *r291* and *sf18* animals grown at 15°C show fewer and thinner needles, compared with animals grown at 20°C. Suppression of *r291* was most complete: there is an almost complete loss of needles. This suppression of the needle phenotype is found even when the shift to lower temperature (for 24 h) was delayed until the young adult (YA) stage (Figure 3, A and B). Interestingly, growth at 15°C did not suppress the motility defect in *unc-96(sf18)* (Figure 3C). Thus, if motility is truly connected to mutations in *unc-96*, the threshold for suppression of the needles phenotype is lower than the threshold for improvement of motility.

Defects in Both Myofibrillar Structure and Motility of *unc-96* Mutants Are Suppressed by Starvation

While conducting experiments, it was noted that when plates of *unc-96* mutants were stored for long periods and the food source (*E. coli*) had been depleted, many animals lacked the characteristic birefringent needles. To explore this phenomenon systematically, we took young adult worms and deprived them of food for 24 h at 20°C. As shown in Figure 3, A and B, this “starvation” resulted in complete loss

of needles and improvement of muscle structure to that of wild type. The improvement in muscle structure also could be seen upon staining with anti-UNC-89 and anti-paramyosin antibodies: the M-lines (UNC-89) and the A-bands (paramyosin) are greatly improved, and distinct accumulations of paramyosin are absent (Figure 3D; compare to animals of the same genotype grown in the presence of abundant food in Figure 2). In addition, it was noted that starvation suppresses the motility defect (Figure 3C): starved *sf18* animals at 20°C move as fast, or even faster, than wild-type animals grown at 20°C. Although starved *sf18* animals were able to recover to wild-type levels of movement, they remained slower than starved wild-type animals. The suppressive effect of starvation was reversible upon refeeding (Figure 3E): 24 h of refeeding restored some of the needles, and 48 h of refeeding restored nearly all of the needles.

Molecular Cloning of the *unc-96* Gene

A combination of three factor and SNP mapping was used to limit *unc-96* to a set of eight overlapping cosmids and one short YAC clone spanning from F38G1 on the left to F02G3 on the right (Figure 4 and *Materials and Methods* for details).

We then tested for the ability of these cosmids to rescue the Unc-96 phenotype when carried as transgenic arrays. One of these eight cosmids, F13C5, gave phenotypic rescue. Next, RNAi was performed for each of the six genes predicted on WormBase as being encoded by the F13C5 sequence. RNAi for one of these genes, F13C5.6, phenocopied the distinctive polarized light “needle phenotype” of an *unc-96* mutant (Figure 1A). This single gene, fused at its 3' end to the coding sequence of GFP, also rescued the Unc-96 mutant phenotype (Figure 1A).

To obtain further evidence that we had identified the *unc-96* gene, we looked for sequence alterations within the F13C5.6 gene among the *unc-96* mutants. Both *r291* and *su151* are G-to-A transitions in highly conserved splice donor sites: for *r291* it occurs at the beginning of intron number 2, and for *su151* it occurs at the beginning of intron number 5. In contrast, *sf18* is a nonsense mutation, a G-to-A transition converting tryptophan 313 (UGG) to the stop codon UGA (Figure 4, bottom). The molecular nature of these mutations seems to be correlated with the motility phenotypes, but not the muscle structural phenotypes (Figure 1). Whereas the splice site mutations, *su151* and *r291* have little to no effect on motility, the termination mutation *sf18*, has a strong effect on motility. The polarized light appearances of all three mutations are indistinguishable. However, by electron microscopy (EM) (Supplemental Figure 1), *r291* shows nearly normal muscle structure, including normal length M-lines. Given the nature of the mutations, it seems that the presence of accumulations (needling phenotype) is more sensitive to the level of *unc-96* gene activity than is muscle structure and motility.

During the course of our RNAi experiments, we noticed that the Unc-96 phenotype could be seen in the P₀ animals that were fed bacteria producing *unc-96* dsRNA. As shown in Figure 1A, young adult animals feeding on the *unc-96* RNAi bacteria for 48 h show the characteristic needles of *unc-96* mutants. During this 48-h period, the young adult matures into an older adult, its body mass increases, and the length and size of its myofibrils increase. Because we observe the *unc-96* mutant phenotype, we know that *unc-96* mRNAs are being degraded. Thus, we conclude that newly synthesized *unc-96* mRNA and protein are required for myofibril assembly in adults.

UNC-96 Exists in Two Isoforms, 408 or 418 Residues, and Has No Recognizable Domains

As predicted on WormBase, and confirmed by our cDNA sequence analysis, there are at least two mRNAs from the *unc-96* gene, designated *unc-96A* and *unc-96B*. These are generated by exclusion or inclusion, respectively, of an alternative exon near the 3' end of the gene, which encodes 10 amino acid residues (Figure 5). The largest UNC-96 polypeptide is 418 amino acids long (Figure 5) and has a calculated molecular mass of 47,886 Da, and a calculated pI of 5.86. The sequence has a reduced percentage of hydrophobic residues (25.6% compared with the average *C. elegans* protein, which is 33.1%) and has an increased percentage of charged residues (35.7% compared with 31.3%). The sequence is particularly enriched for aspartate at 8.4%, proline at 7.9%, arginine at 9.6%, and serine at 11.5%, compared with the average *C. elegans* protein, which has aspartate at 5.4%, proline at 5.0%, arginine at 5.3%, and serine at 8.1%. Computer programs have failed to reveal any recognizable protein domains in the UNC-96 sequence. Nevertheless, BLAST searches have revealed high level, nearly exact matches, spanning 13 or 14 residues between UNC-96 and two mammalian pro-

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MSETEDVNLNLDPEKPKVRSVVRSDSGLENEIPKISGTSALIEEGLSLL
NLKSSLSNPVAPVAVDLDLRTCPSPDYGLTSERLASVKTPRFKRIDFDDR
LEDLNPKNPAWKSQENIKDSYHKVKSAAKKREDNEFMDNMLGRARPI
RGESPYRNLSLDTGFIPTSVRSRSSIMGPSLSDRSRLATSSSTTNMFNAGS
GPYAAPALTISQIGESRYEERANNVELMLLKNAPLPERYKSITTFRRRA
PEPSAGSFSEQDDYDFSHTTARPYYSRPNRDDPDYDFDLQHSVDMFKR
PEGRYIPRKPEWENKLISESTGKGTAPLSGHMFTRPDTWRNNNTSYLS
AALRTPKFWEQRFENIGKHVRDNPISLESINFRIRTLQDIRANRPVTSR
FTEYRDPDYYDYEDPLDD 418aa
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nearly identical to a portion of Hs ZNF480
 nearly identical to a portion of Hs FLJ00128
 alternative splicing

SVKTPRFKRIDFDD UNC-96
 SVKTP F R DFDD
 SVKTFIPNRNDFDD Hs ZNF480
 Identities = 78%; Positives = 78%

RRAPEPSAGSFSE UNC-96
 RRAPEPSAG+F E
 RRAPEPSAGTFQE Hs FLJ00128
 Identities = 84%; Positives = 92%

Figure 5. Amino acid sequence of UNC-96. High-level, nearly exact matches spanning 13 or 14 residues were found to two mammalian proteins of unknown function (Hs FLJ00128, accession no. BAB84883 and Hs ZNF480, accession no. AAR97581). Note that two isoforms of UNC-96, A and B, differ by 10 residues, boxed in red, and are encoded by an alternatively spliced exon (confirmed by cDNA sequences). Highlighted are residues each of which are represented 3–4% higher than in the average *C. elegans* protein (D, P, R, and S).

teins of yet unknown function (Figure 5). Similarity spans across species, especially for the sequence similar to the human sequence Hs FLJ00128, which is found in five other mammals. Perhaps these short stretches of homology reflect conserved structures, or binding sites, not yet assigned functional significance.

Anti-UNC-96 Reacts with a Polypeptide of Expected Size from Wild Type, but This Protein Is Absent or in Reduced Amounts in *unc-96* Mutants

We generated and affinity-purified rabbit antibodies to bacterially expressed His-tagged, full-length UNC-96 protein. As shown in Figure 6, these anti-UNC-96 antibodies (EU148) react to a polypeptide of ~47 kDa from wild-type total soluble proteins on an immunoblot. The size of this polypeptide is very close to the size of the UNC-96 protein predicted from sequence analysis (47,886 Da). At the same exposure, no anti-UNC-96 reacting proteins were detected in extracts prepared from all three *unc-96* mutants (equally loaded relative to wild type). However, after a long exposure to film, we could detect several protein bands from *r291*, one of which (indicated by an arrow) is the same size as the protein detected from wild type. This is interpreted as a small amount of full-length UNC-96 protein expressed in this splice donor mutant, consistent with near normal muscle structure by EM (Supplemental Figure 1). (The other bands were also present in *su151* and *sf18* on longer exposure [our unpublished data]. These likely represent low-level cross-reactivity to other *C. elegans* or even *E. coli* proteins.)

Localization of UNC-96 by Using Antibodies and a GFP Fusion

To gain further insight into the function of the UNC-96 protein, we determined where the protein is normally lo-

Figure 6. By Western blot, anti-UNC-96 antibodies detect a polypeptide of expected size from wild type (wt), which is absent or in reduced amounts from *unc-96* mutants. Western blot of whole worm extracts from wild type (wt), *r291*, *su151*, and *sf18* reacted with affinity-purified antibodies to UNC-96 shows an ~47-kDa polypeptide (expected size) from wild type only. The black horizontal lines with numbers denote the positions of molecular weight markers of the indicated sizes in kilodaltons. The lane to the left of the marker lane depicts results from *unc-96(r291)* after a longer exposure to film; note the presence of a band at the same size as detected from wild type. This is interpreted as a small amount of full length UNC-96 protein expressed in this splice donor mutant, *r291*. The other bands are presumed to be cross-reacting, because they are also seen in wt, *su151*, and *sf18* at this longer exposure (our unpublished data).

cated in muscle. Anti-UNC-96 antibodies (EU148) were used to localize UNC-96 in adult wild-type worms using immunofluorescence microscopy. As shown in Figure 7A, in body wall muscle, UNC-96 is located in M-lines, identified by use of antibodies to the M-line marker UNC-89. UNC-96 and UNC-89 are also similarly expressed in pharyngeal and anal depressor muscles (Figure 7A). In such single sarcomere muscles, UNC-96 is found in the middle of the A-bands, a location similar to that found in body wall muscles. We carefully examined the pharyngeal muscle structure of all three *unc-96* mutant alleles by polarized light but were unable to detect any abnormalities. Staining of the *unc-96* mutants with the anti-UNC-96 antibodies revealed the presence of UNC-96 in the pharynx for *r291* only. For all the alleles, no staining was detected in the body wall muscles. This result is consistent with our Western data showing that *r291* likely produces a low level of full-length UNC-96.

We generated transgenic worms expressing full-length UNC-96 fused at its C terminus to GFP, as directed by ~2 kb of sequence upstream of the *unc-96* coding sequence. As noted above, this transgene was able to rescue the *Unc-96* mutant phenotype and is thus likely to reflect the true expression and localization of the endogenous protein. In accordance with our antibody results, *unc-96::gfp* is expressed in body wall and pharyngeal and anal depressor muscles (Figure 7B; anal depressor expression not shown). In the single sarcomere muscles, UNC-96::GFP is located in the middle of A-bands. In body wall muscle, UNC-96::GFP is specifically located at M-lines, and in addition, at dense bodies. UNC-96::GFP could be seen as early as the 1.5-fold embryonic stage (our unpublished data).

UNC-96 Is Expressed in Embryonic Body Wall and Pharyngeal Muscle

Embryos were costained with anti-UNC-96 and anti-MHC A. As shown in Figure 8, UNC-96 is first detectable at the 1.5-fold stage, in which UNC-96, like MHC A, is diffusely localized in the cytoplasm of body wall muscle cells. It has

A anti-UNC-96 localization

B UNC-96::GFP localization

Figure 7. Localization of anti-UNC-96 antibodies and UNC-96::GFP in adult muscles. (A) Localization of anti-UNC-96 antibodies in adult body wall and pharyngeal and anal depressor muscle. The top three panels show portions of two body wall muscle cells simultaneously stained with anti-UNC-96 and a known marker of M-lines, anti-UNC-89. UNC-96 resides in M-lines, but it does not seem to completely colocalize with UNC-89. The fourth panel shows that UNC-96 is expressed in the pharyngeal muscles (anterior, right; posterior, left). The fifth panel shows optical cross sections through a worm at the level of the terminal bulb of the pharynx stained with anti-UNC-96 and anti-UNC-89. The outer ring of staining is from the four body wall muscle quadrants, and the inner ring of staining is from the terminal bulb muscles. As shown previously for UNC-89, UNC-96

Figure 8. Localization of anti-UNC-96 antibodies in embryonic muscles. Embryos were costained with anti-UNC-96 and anti-MHC A. As shown in the top panel, UNC-96 is first detectable at the 1.5-fold embryonic stage, in which UNC-96, like myosin heavy chain A, is localized to the cytoplasm of body wall muscle cells. As shown in the bottom panel, by the threefold stage, UNC-96 is almost undetectable in body wall muscle cells, but it is prominently found in the pharyngeal muscles.

been established that at the 1.5-fold stage, nascent linear structures containing MHC A, MHC B, and paramyosin are present, but myofibrils have not yet formed (Epstein *et al.*, 1993). By the threefold stage, UNC-96, with the use of our antibodies, is undetectable in body wall muscle, but it can be seen very prominently in embryonic pharyngeal muscle (Figure 8).

unc-96 and unc-98 Have Distinct, Nonredundant Functions That Converge on the Same Endpoint for Myofibril Organization

As first reported by Zengel and Epstein (1980), the polarized light phenotypes of *unc-96* and *unc-98* mutants are nearly identical. This suggests that the two genes may participate in the same biological function, or interact functionally. As shown in Figure 9, the polarized light phenotype of loss-of-function for *unc-96* (phenocopied by RNAi) is identical to that of *unc-98(sf19)*. We sought to determine the phenotypic outcome of animals having reduced activity for both *unc-96* and *unc-98*. To do this, the F1 generation of *unc-98(sf19)* animals, fed bacteria expressing dsRNA for *unc-96*, were examined by polarized light microscopy. As shown in Figure 9, *unc-98(sf19); unc-96(RNAi)* animals are not different in phenotype from either single mutant. The reciprocal experiment was also done: *unc-96(sf18)* animals were fed bacteria expressing dsRNA for *unc-98*. The resulting F1 *unc-96(sf18); unc-98(RNAi)* animals also showed no obvious enhancement or suppression over the single mutants (our unpublished data). These data suggest that the two genes are likely to reside in the same linear pathway, or parallel pathways that converge on the same end point.

Figure 7 (cont). also localizes to the middle of the single sarcomere pharyngeal muscles. The sixth panel shows the tail region of a worm costained with antibodies to UNC-96 and UNC-89. UNC-96 resides in the middle of the single sarcomere anal depressor muscles (one dorsal, one ventral). (B) Localization of UNC-96::GFP. Top, body wall muscle; bottom, pharyngeal muscle. In body wall muscles, UNC-96::GFP is located at M-lines, like the antibodies, but, in addition, is also located at dense bodies. In pharyngeal muscles, UNC-96::GFP is localized similarly to the localization of UNC-96 antibodies.

To determine whether the overexpression of *unc-98* can suppress the *unc-96* mutant phenotype, we expressed an *unc-98::gfp* transgene in an *unc-96(sf18)* mutant background. Because the endogenous *unc-98* gene is also present, such animals are overexpressing *unc-98* and thus can be considered gain-of-function for *unc-98*. This *unc-98::gfp* construct has two properties: 1) it can rescue the phenotype of *unc-98* mutants (Mercer *et al.*, 2003); and 2) in a wild type background, expression of this *unc-98::gfp* transgene results in worms with normal muscle structure by polarized light. We also did the reciprocal experiment in which *unc-96::gfp* was overexpressed in an *unc-98(sf19)* mutant background. To our surprise, in each case the needle phenotype was enhanced in terms of the size and intensity of the needles (Figure 9A). The muscle structures were also more disorganized compared with either single mutant. We conclude that overexpression of one of the proteins is not sufficient to compensate for the absence of the other protein, and thus UNC-96 and UNC-98 are not redundant.

Consistent with the idea that *unc-96* and *unc-98* interact genetically, we found that, antibodies against UNC-98 stain distinct accumulations in *unc-96* mutants, and antibodies against UNC-96 stain accumulations in *unc-98* mutants (Figure 9B). In each case, the location (ends of muscle cells) and shape of these accumulations is similar to the birefringent needles seen by polarized light in each mutant.

UNC-96 Interacts with UNC-98 by Yeast Two-Hybrid Assay and In Vitro

Because *unc-96* and *unc-98* interact genetically, we sought to determine whether the two proteins are able to interact biochemically. We performed a yeast two-hybrid assay by using varying length constructs of UNC-96 as bait to evaluate the ability of the truncated proteins to interact with full-length UNC-98 (as the prey). We found that the C-terminal half of UNC-96 (201-418 aa) was capable of an interaction with full-length UNC-98. A nearly full-length product of UNC-96 (45-418 aa), which contains the aforementioned C-terminal region, was unable to interact with full-length UNC-98 (Figure 10A). This suggests that the N-terminal half of UNC-96 may inhibit the interaction between UNC-96 and UNC-98, in this assay. Next, we investigated which portions of UNC-98 were important for this protein-

Figure 9. Genetic interaction between *unc-96* and *unc-98*. (A) Polarized light microscopy of body wall muscle from animals with the indicated genotypes. The top panel shows muscle from an animal that has undergone RNAi for *unc-96*. The second panel shows muscle from *unc-98(sf19)*. Loss of function for *unc-98* results in a phenotype nearly identical to *unc-96*. The third panel shows an *unc-98(sf19)* worm that has undergone RNAi for *unc-96*. Note that in this animal, there is no worsening in phenotype as compared with loss-of-function for *unc-96* or *unc-98*, alone. The fourth panel shows an *unc-96(sf18)* animal that is expressing UNC-98::GFP from a transgene. The fifth panel shows an *unc-98(sf19)* worm that is expressing UNC-96::GFP from a transgene. Note that there is worsening of phenotype (greater degree of myofibril disorganization, larger and brighter needles) in either *unc-96(sf18)* animals with increased expression of UNC-98, or *unc-98(sf19)* animals with increased expression of UNC-96. (B) Immunofluorescence localization of UNC-98 and UNC-96. Distinct accumulations at the ends of muscle cells in *unc-96* mutants contain UNC-98 protein (top). Similarly, accumulations in *unc-98* mutants contain UNC-96 protein (bottom).

protein interaction. The data suggest that all four zinc fingers of UNC-98 are necessary and sufficient for the strongest interaction between UNC-98 and UNC-96 (201-418 aa) (Figure 10B, construct A). Additionally, the nonzinc finger N-terminal region of UNC-98 is not necessary for interaction. The last three zinc fingers were sufficient for a weaker level interaction (as evident by low-level yeast growth, construct

Figure 10. UNC-96 interacts with UNC-98 by yeast two-hybrid and far Western assays. (A) Mapping of the binding site in UNC-96 for UNC-98. The UNC-96 protein is represented as a long yellow colored rectangle with amino acid numbers beneath. The three rows of rectangles represent the portions of UNC-96 that were tested for binding to UNC-98 (full-length) by the two-hybrid assay. The green colored rectangle represents the smallest fragment that showed binding to UNC-98. "Binding to UNC-98 (full-length)" columns show results of binding. A + means growth on the selection media, and - means no growth on the selection media. (B) Mapping of the binding site in UNC-98 for UNC-96. The UNC-98 protein is represented as a long rectangle with amino acid numbers beneath. The red and orange boxes denote the C2H2 zinc fingers, green boxes denote predicted NLS, and blue boxes denote predicted nuclear export signal sequences (one lies within the first zinc finger). The five rows of rectangles represent the portions of UNC-98 (Mercer *et al.*, 2003) that were tested for binding to UNC-96 (201-418) by the two-hybrid assay. The green-colored rectangles represent the fragments that showed strong binding to UNC-96 (201-418), and yellow-green-colored rectangle represents the fragment that showed weak binding to UNC-96 (201-418). The "Binding to UNC-96 (201-418)" columns show results of binding. A + means growth on the selection media, ± means weak growth on the selection media, and - means no growth on the selection media. Photos of yeast cells show growth on selection media (three independent colonies are shown for each). (C) The C-terminal half of UNC-96 interacts with UNC-98 by the far Western assay. On the right is a Coomassie-stained SDS-PAGE of the starting materials, full-length UNC-98 with a 6His tag (98His), MBP, and UNC-96 (201-418) fused to MBP (96MBP). On the left is the far Western: a gel similar to the one on the right

Figure 11. *unc-96* and *unc-15* interact genetically. UNC-96 and paramyosin interact biochemically. (A) The homozygous mutant, *unc-15(e1215); unc-96(sf18) unc-1(e538)* shows an enhanced phenotype compared with the single mutants, *unc-15(e1215)*, *unc-96(sf18) unc-1(e538)*, and *unc-15(e1215); unc-1(e538)*. *unc-15(e1215)*, *unc-96(sf18) unc-1(e538)*, and *unc-15(e1215); unc-1(e538)* are slightly slower than wild type. As shown in panel 2, the double mutant *unc-15(e1215); unc-96(sf18) unc-1(e538)* is completely paralyzed in an unusually folded posture. Bar, 0.1 mm. (B) UNC-96 is mislocalized in body wall muscle cells lacking paramyosin. The top panel shows the localization of UNC-96 to M-lines in wild-type muscle. The bottom panel shows that UNC-96 is weak and diffuse in muscle from the paramyosin null mutant *unc-15(e1214)*. Staining of α -actinin, a major component of dense bodies, was used to locate muscle cells due to the very weak staining of anti-UNC-96 in the paramyosin null. Some secondary disorganization of dense bodies can be seen in *unc-15(e1214)*. Bar, 5 μ m. (C) By ELISA, UNC-96 binds to paramyosin in a saturable manner. The left two panels show a Coomassie-stained SDS-PAGE of purified paramyosin (PM) and bacterially expressed His-tagged UNC-96 with Western blotting. On the right is a plot of the ELISA. Because paramyosin was exposed to higher concentrations of UNC-96, from 0 to 0.5 μ M, it bound increasingly until it reached near saturation. UNC-96 did not bind BSA within this concentration range.

B). However, loss of either zinc finger 2 (construct C) or zinc finger 4 (construct D), independently, resulted in a lack of

interaction. Because neither zinc finger alone was sufficient for an interaction, the results underline the importance of having both, and possibly the third, zinc fingers to obtain an interaction between the two proteins.

To confirm the results of the two-hybrid assay, we tested whether bacterially expressed UNC-96 and UNC-98 could be shown to interact directly in a far Western assay. Full length UNC-98 with a 6His tag (98His) was run on SDS-PAGE, transferred to membrane, and incubated with UNC-96 MBP fusion protein (96MBP), which contains the same portion of UNC-96

Figure 10 (cont). was used to separate 98His, and the protein was transferred to a membrane. Next, one blot strip was incubated with MBP, and another was incubated with 96MBP, washed, and each was incubated with antibodies to MBP conjugated to horseradish peroxidase (anti-MBP/HRP). Reactions were visualized by ECL. Note that 96MBP but not MBP interacts with 98His.

Figure 12. UNC-96 copurifies with nematode thick filaments. (A) As shown in the schematic diagram, the process of thick filament purification involves Triton X-100 extractions, Dounce homogenization, and differential centrifugation. Fractions were collected throughout the purification process (F1-14). (B) The fractions (F1-14) were separated on SDS-PAGE, blotted, and reacted to anti-UNC-96 antibody. Notice that UNC-96 copurifies with thick filaments in fraction F14. (C) Fraction F11 was further separated on a sucrose gradient. Fractions from this gradient (S1-14, from bottom to top) were pooled: S1-5, S6-10, and S11-14. Portions of each pool were separated on two gels, one gel for Coomassie staining, and the other gel for Western blotting. Coomassie staining of the pooled proteins shows that fractions S1-5 contain myosin (MHC) and PM, fractions S6-10 contain a small amount of actin (ACT) and tropomyosin (TM), and fractions S11-14 contain ACT and TM. Reaction of the UNC-96 antibody against a Western blot shows that UNC-96 is present in the fractions containing both myosin and paramyosin. Given myosin and paramyosin are the primary components of thick filaments, this indicates that UNC-96 copurifies with thick filaments.

(residues 201-418) that gave the strongest interaction with UNC-98 in the two-hybrid experiments. A comparable blot strip of 98His was also incubated with MBP as a control. The binding reactions were visualized using antibodies to MBP followed by ECL. As shown in Figure 10C, 96MBP but not MBP reacts to 98His on the blot. In addition, full-length UNC-96 fused to MBP also reacted to 98His (our unpublished data). In further support of the far Western results, we note that in a glutathione *S*-transferase (GST) pull-down assay, more 96MBP was pulled down with full-length UNC-98 fused to GST, compared with GST alone (our unpublished data).

unc-96 and *unc-15* Interact Genetically; UNC-96 and Paramyosin Interact Biochemically

Given the localization of paramyosin to accumulations in *unc-96* mutants (Figure 2), we sought to determine whether UNC-96 and paramyosin interact. We found that *unc-96* and *unc-15*, the structural gene for paramyosin, interact genetically both as trans-heterozygotes and as double homozygotes. For this purpose, we used a mild allele of *unc-15*, *e1215*. Although *unc-96(sf18)/+* has a normal muscle structure by polarized light, and *unc-15(e1215)/+* has only a mildly disorganized muscle structure by polarized light, the compound heterozygote, *unc-15(e1215)/+; unc-96(sf18)/+*, displays enhanced disorganization of muscle structure, similar to that of the *unc-96(sf18)* homozygote (Supplemental Figure 2). *unc-15(e1215)* homozygotes are significantly

slower than wild type but show normal posture. *unc-96(sf18)* homozygotes are slightly slower than wild type and also show normal posture. However, the double mutant, *unc-15(e1215); unc-96(sf18)* is completely paralyzed and has an abnormal "folded-up" posture (Figure 11A). This folded paralysis is seen beginning at the L1 larval stage, but growth continues in the same paralyzed state until adulthood. We next reasoned that if UNC-96 normally interacts with paramyosin, then in the absence of paramyosin, UNC-96 localization should be disrupted. As shown in Figure 11B, in muscle from the *unc-15* null allele, *e1214*, UNC-96 is not localized to the M-lines and is present, diffusely, at low levels in the muscle cells. Because of our results showing an interaction between paramyosin and UNC-96 in vivo, we wondered whether a direct interaction could be demonstrated in vitro. For this purpose, we purified paramyosin from wild-type worms and expressed full-length UNC-96 as a His-tagged protein (Figure 11C, left). In an ELISA experiment, we could demonstrate saturable binding of UNC-96 to paramyosin but not to BSA (Figure 11C, right).

UNC-96 Copurifies with Nematode Thick Filaments

Significant mislocalization of paramyosin in *unc-96* mutants, and the interaction between UNC-96 and paramyosin, prompted us to investigate the possible association of UNC-96 with thick filaments. Also, because UNC-96 localizes to the M-line, a structure in which thick filaments are

cross-linked, a direct association with thick filaments seemed likely. We prepared native thick filaments from *C. elegans* by using established procedures (Epstein *et al.*, 1988; Deitiker and Epstein, 1993) and tested each step of the thick filament preparation (Figure 12A) with anti-UNC-96 on a Western blot. As shown in Figure 12B, UNC-96 occurs in the 15K pellet containing thick filaments and low levels of thin filaments, ribosomes, and nuclear fragments. This indicates that UNC-96 may be associated with thick filaments. Further purification of these thick filaments by sucrose gradient sedimentation of the 5K supernatant material shows that UNC-96 follows known markers of the thick filament, namely, myosin heavy chains and paramyosin (Figure 12C).

DISCUSSION

Study of three loss-of-function mutant alleles has shown that there are two aspects to the *unc-96* phenotype: protein accumulations ("needling") at the ends of muscle cells and myofibril disorganization. The muscles of all three alleles, when viewed by polarized light, exhibit an equivalent needling phenotype. However, EM reveals that the three alleles differ in their degree of myofibril disorganization, with *r291*, unlike *su151* and *sf18*, having nearly wild-type myofibril structure. A third potential aspect of the phenotype is reduced motility, which may correlate with the structural defects seen by EM. Additional mutant alleles will be required to determine whether indeed reduced motility is a defect associated with mutations in *unc-96*. We have observed that the needling phenotype can sometimes be seen in the presence of normal myofibril structure (e.g., P₀ exposure to F13C5.6 RNAi, 48 h; Figure 1), evidence that the needling/protein accumulation phenotype is more sensitive than is the structural defect to levels of UNC-96. This may indicate that *unc-96* is important for a function in addition to myofibril organization.

Several pieces of intriguing data indicate that UNC-96 has a role in adult muscle: 1) The suppressive effect of starvation on the *Unc-96* mutant phenotype is reversible upon reexposing adults to plentiful food. 2) The *Unc-96* phenotype can be observed in young adult animals fed with *unc-96* RNAi bacteria for 48 h. 3) The suppressive effect of reduced temperature (15°C) on the *Unc-96* mutant phenotype can be observed even when the exposure to lower temperature begins at the young adult stage. This suggests that UNC-96 is continuously required in adult muscle for the final stages of sarcomere assembly and possibly maintenance of already established myofibrils.

Antibodies generated to UNC-96 show that the protein is located at the M-lines in adult body wall muscle and in the middle of A-bands in the single sarcomere pharyngeal and anal depressor muscles. An *unc-96::gfp* fusion that rescues *unc-96* mutants is expressed in the same cells that anti-UNC-96 staining is detected. However, in body wall muscle, in addition to being localized to M-lines, the GFP fusion protein is also located at dense bodies. This phenomenon is similar to that observed for UNC-98 (Mercer *et al.*, 2003). There are several possible reasons for the discordance between localization obtained with the anti-UNC-96 antibody and UNC-96::GFP fusion. The most likely explanations are that either the dense bodies are inaccessible to the antibodies or that UNC-96 is poorly fixed in dense bodies. Another possibility is that indeed UNC-96 is normally present in dense bodies but at such a low concentration that it is undetectable by immunofluorescence. This is consistent with our impression that the total concentration of UNC-96 in body wall muscle is low (e.g., even at 1:100 dilution the

staining of M-lines is "weak"). Our anti-UNC-96 antibodies are not likely to be low titer given the strong pharyngeal staining. Despite some similarities in localization to UNC-98 (Mercer *et al.*, 2003) and UNC-97 (Hobert *et al.*, 1999), we found no antibody or GFP fusion protein evidence for UNC-96 being located in muscle cell nuclei.

Given that we have localized UNC-96 to the M-line region at the light microscope level and that EM images of vertebrate sarcomeres show that the M-line is a region in which thick filaments are cross-linked by "M-bridges" (Knappes and Carlsen, 1968; Luther and Squire, 1978), it is not surprising that UNC-96 copurifies with thick filaments. One possibility is that UNC-96 might bind along the shaft of the thick filament and then oligomerize to form M-bridges.

Our genetic and biochemical data show that UNC-96 interacts with paramyosin (Figure 11). The current model for the nematode thick filament shows it to be a series of concentric layers: an outer layer composed of MHC A and B, an intermediate layer of paramyosin, and an inner or core layer of paramyosin and the filagenins (e.g., Deitiker and Epstein, 1993). Because paramyosin is apparently buried under an outer layer of the myosins, it is difficult to understand how interaction between UNC-96 and paramyosin permits association of UNC-96 with the shaft of the thick filament. One clue is provided by our localization of paramyosin in *unc-96* mutants. As shown in Figure 2, although some paramyosin is found in accumulations, much of it is also found localized to A-bands. We propose that UNC-96 normally has two locations within muscle cells: some of it is a fixed component of the M-line (which may be associated with paramyosin), and some of it is associated with paramyosin that has either dissociated from, or has not yet been incorporated into, thick filaments. Thus, UNC-96 may facilitate the degradation of paramyosin that has dissociated from thick filaments and/or facilitate the incorporation of newly synthesized paramyosin into thick filaments. Through either mechanism, UNC-96 would be promoting myofibril maintenance, such that when UNC-96 is deficient, paramyosin accumulates abnormally. Interestingly, we found that starvation of *unc-96* mutant worms can cause the paramyosin accumulations to disappear, and the overall myofibrillar structure to improve. There are at least two possible explanations for the effects of starvation. In the case of vertebrate muscle, acute starvation is known to elevate the overall breakdown of myofibrillar proteins through the up-regulation of the ubiquitin/proteasome system (UPS; Mitch and Goldberg, 1996). Thus, an up-regulated UPS might be able to degrade the paramyosin that accumulates in *unc-96* mutants. Alternatively, we can postulate that there is a competition between incorporation of paramyosin into needles versus incorporation into thick filaments, and the major function of UNC-96 is to promote incorporation of paramyosin into thick filaments. Thus, starvation is an alternative way (by, as yet, an unknown mechanism) by which, in the absence of UNC-96 activity, incorporation of paramyosin into filaments is favored.

A role for an M-line protein in maintaining the structure of contracting myofibrils is not without precedence. Two examples from mammalian muscle are the M-line portion of the giant protein titin, and the RING finger protein MURF-1. The M-line portion of titin contains its protein kinase domain that is likely to be activated repeatedly by the force of muscle contraction (Grater *et al.*, 2005). Lange *et al.* (2005) have shown that associated with the titin kinase is a set of proteins, including nbr1, p62, MURF-2, and SRF. The phosphorylation of nbr1 results in less MURF-2 and more SRF in nuclei, leading to increased expression of SRF-driven genes, which include myofibril proteins. Indeed, Lange *et al.* (2005)

suggest that this is probably how muscle activity maintains myofibril mass, and how muscle inactivity leads to muscle atrophy. The progressive muscle wasting and gradual sarcomere disassembly seen in both a hereditary myopathy due to missense mutation in the regulatory region of titin kinase in humans (Lange *et al.*, 2005) and a conditional knockout of the M-line region of titin in mice (Gotthardt *et al.*, 2003) support this model. MURF-1 was originally identified as a binding partner of M-line titin, and its RING finger is likely to have ubiquitin ligase activity (Centner *et al.*, 2001). Significantly, MURF-1 is up-regulated during muscle atrophy, and mice deficient in MURF-1 are resistant to atrophy (Bodine *et al.*, 2001). In *C. elegans*, a titin-like protein is not found at the M-line (Flaherty *et al.*, 2002), and MURF orthologues may not exist (Centner *et al.*, 2001). This raises a question as to whether a similar mechanism exists in *C. elegans* muscle. Nevertheless, given what we now know about UNC-96, it is possible that UNC-96 may be involved, together with interacting proteins, in analogous signaling networks to promote turnover of muscle proteins. Additionally, in *C. elegans*, RNF-5, a RING finger protein with demonstrated ubiquitin ligase activity (Didier *et al.*, 2003), interacts with and regulates the levels of UNC-95 protein at the dense body (Broday *et al.*, 2004).

Our genetic, two-hybrid and in vitro binding data also indicate that UNC-96 is associated with UNC-98, another M-line protein (Mercer *et al.*, 2003). Two-hybrid data show that this interaction occurs between the C-terminal half of UNC-96 (residues 201–418), and C2H2 zinc fingers 1–4 or 2–4 of UNC-98. It is noteworthy that the same portion of UNC-98, that is, zinc fingers 1–4, has also been shown to interact with UNC-97 (Mercer *et al.*, 2003), a protein that is composed of 5 LIM domains and is required for M-line and dense body assembly (Hobert *et al.*, 1999). Either this portion of UNC-98 is capable of simultaneously interacting with both UNC-97 and UNC-96, or there are mutually exclusive complexes of UNC-98/UNC-97 and UNC-98/UNC-96. Although questions remain regarding the composition of protein complexes that include UNC-96, it is clear by our genetic data that *unc-96* and *unc-98* have distinct, nonredundant functions that converge on the same endpoint. Because the accumulations in *unc-96* mutants contain both paramyosin and UNC-98, and *unc-96* and *unc-98* reside in the same linear pathway, we speculate that they function together in the turnover of thick filament components such as paramyosin.

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