Maternal UNC-45 is involved in cytokinesis and colocalizes with non-muscle myosin in the early *Caenorhabditis elegans* embryo

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Summary

The *Caenorhabditis elegans* UNC-45 protein contains tetratricopeptide repeats and a domain with similarity to fungal proteins, and it differentially colocalizes with myosin heavy chain B in the body wall muscles of adult worms. Although it is essential for normal myosin filament assembly in body wall muscle development, strong mutants show a previously unexplained maternal effect. We show here that the UNC-45 protein is maternally contributed and is present in all cells of the early embryo whereas zygotic UNC-45 expression is only detected in the developing muscle cells. Embryos produced from adults with reduced germline expression of UNC-45 exhibit cytokinesis defects suggesting that UNC-45 has a novel role in the early embryo in addition to muscle development. Yeast two-hybrid screens show that UNC-45 can directly interact with NMY-2, a non-muscle type II myosin, and UNC-45 and NMY-2 colocalize at cell boundaries in early embryos. Localization of UNC-45 at these boundaries is dependent upon the presence of NMY-2. Our results suggest that UNC-45 interacts with more than one type of myosin and functions in the embryo to regulate cytoplasmic myosin assembly and/or stability during cytokinesis.

Key words: Myosin heavy chain, UCS domain protein, Cytokinesis, Two-hybrid assay, Non-muscle myosin

Introduction

Although myosin has an intrinsic affinity to assemble in vitro and in some in vivo systems, in the absence of extracts from myogenic cells the head domain does not fold properly; therefore, other factors must be involved in folding the myosin head in vivo (Barral and Epstein, 1999; Srikakulam and Winkelmann, 1999; Hutagalung et al., 2002; Chow et al., 2002). Chaperones shown to promote proper myosin folding include UNC-45 (Barral et al., 2002), and a similar function is proposed for proteins from other systems that share sequence similarity in the C-terminal domain of UNC-45 (Hutagalung et al., 2002), recently termed the UCS (UNC-45/CRO-1/She4p) family of proteins. UNC-45 is required in the developing nematode *Caenorhabditis elegans* for normal assembly of the myosin-containing thick filaments in the body wall muscles. The *unc-45* gene was first identified through a temperature sensitive (*ts*) viable allele that results in a paralyzed phenotype due to disorganized thick filaments of the body wall muscles (Epstein and Thomson, 1974). These muscles contain both major (MHC B) and a minor (MHC A) isoforms of type II myosin heavy chain (65% identical and 79% similar), encoded by different genes (*unc-54* and *myo-3*, respectively). The UNC-45 protein immunostaining pattern in these cells is identical to the pattern seen with MHC B, but not MHC A, in larval and adult animals, and UNC-45 no longer associates with thick filaments that are composed exclusively of MHC A (Ao and Pilgrim, 2000). The activity of UNC-45 that is affected in the *unc-45(ts)* alleles is dispensable in the absence of the MHC-B isoform. There is evidence from in vitro studies that UNC-45 can interact with myosin in pull-down assays (Barral et al., 2002) suggesting that the colocalization of MHC B with UNC-45 in vivo may be caused by a direct interaction. These observations have led to the conclusion that UNC-45 acts directly to ensure proper folding of MHC B in the body wall muscles. Although it is possible that UNC-45 interacts with other muscle myosins, an interaction with MHC B in the body wall muscle is sufficient to account for the adult paralysis phenotype.

In addition to the effect on MHC B in the larval and adult body wall muscle, UNC-45 must have another role during embryonic development. Null alleles of *unc-54* are paralyzed but viable and fertile (Epstein and Thomson, 1974). In contrast, nonsense alleles of *unc-45* result in embryonic lethality (Venolia and Waterston, 1990; Barral et al., 1998). Embryos homozygous for the strongest lethal alleles lack any muscle contractions during embryogenesis and arrest before embryonic elongation is complete: a paralyzed-arrest at twofold (Pat) phenotype (Venolia and Waterston, 1990; Venolia et al., 1999). Thus, the phenotype of embryos carrying strong *unc-45* alleles is much more severe than the phenotype of embryos lacking MHC B. *unc-54* null mutations do not suppress the *unc-45* embryonic lethality, as would be expected if the only role of UNC-45 was to control the assembly or stability of MHC B (Venolia and Waterston, 1990). As we have previously noted,

UNC-45 may have a role with MHC A (encoded by *myo-3*) in the initiation of body wall muscle thick filament assembly (Ao and Pilgrim, 2000), as *myo-3* null alleles are also embryonic lethal with a 'Pat' phenotype. This leads to the possibility that UNC-45 may be essential for proper MHC A assembly or function during embryonic development, although there is as yet no direct evidence to support this.

Alternatively, the embryonic effect may not be related to muscle at all. Muscle cell components are normally produced zygotically, but embryonic-lethal alleles of *unc-45* show terminal phenotypes that are dependent on the genotype of the mother, suggesting that *unc-45* product is contributed through the oocyte (Venolia and Waterston, 1990). As the embryos examined in that work were either produced by heterozygous mothers (and may therefore include a dowry of wild-type protein contributed via the oocyte), or were homozygous for alleles that may not be null, the authors would not have been able to unambiguously test whether maternal UNC-45 was necessary for early embryogenesis (Venolia and Waterston, 1990). *C. elegans* non-muscle type II myosins are known to play an essential role in embryonic cytokinesis (Guo and Kemphues, 1996; Shelton et al., 1999), and it is tempting to speculate that UNC-45 may interact with non-muscle myosin molecules in this process. This could be tested by examining cytokinesis in embryos depleted for UNC-45. Indeed, UNC-45 shares sequence similarity with the fission yeast Rng3 protein, which is required for proper cytokinesis and has been shown to interact with a type II myosin, Myo2p (Wong et al., 2000). Another UCS protein that interacts with Type I and V myosins is She4p (Wesche et al., 2003; Toi et al., 2003). Mammalian UCS proteins have also been proposed to have a role during cytokinesis (Price et al., 2002). Therefore, we examined the interaction of UNC-45 with non-muscle myosin II (NMY-2) and characterized the embryonic requirement for UNC-45 during cytokinesis.

Materials and Methods

Strains and genetics

C. elegans strain N2 (wild type) was obtained from the stock collection of the MRC Laboratory of Molecular Biology, Cambridge, UK and maintained as described (Wood, 1988). The alleles of *unc-45* have been described previously (Venolia and Waterston, 1990; Barral et al., 1998; Ao and Pilgrim, 2000). The *hum-2* deletion strain RB801 *hum-2(ok596)* was generated as part of the *C. elegans* Gene Knockout Consortium, and was a gift of Robert Barstead (Oklahoma Medical Research Foundation, Oklahoma City, OK). The yeast strain PJ69-4A along with the expression vectors for the two-hybrid screen were kindly provided by Philip James (University of Wisconsin, Madison, WI) and maintained as described (James et al., 1996). All yeast transformations were done using the high efficiency lithium acetate method (Gietz and Schiestl, 1995).

Transgenic animals

The construct (pDP#WA036), which contains the full-length *unc-45* cDNA fused in-frame to green fluorescent protein (GFP) behind the *unc-45* promoter, was described previously (Venolia et al., 1999). The transgenic line (DP193 *edEx74*) generated as described (Mello et al., 1991) contains this construct as part of an extrachromosomal array along with the plasmid pRF4 [*rol-6(su1006dm)*]. An isolate of this line showing transmission of the array to more than 95% of the progeny was used in this study. GFP expression was examined using the FITC (fluorescein isothiocyanate) filter set on an Axioskop (Carl Zeiss) microscope.

In a second line, homozygous *unc-45(r450ts)* animals were injected with a mixture of plasmids pDP#WA036 and a *myo-3p*::GFP marker supplied by Andy Fire (pPD118.20) (Carnegie Institute of Washington, Baltimore, MD) and scored for *unc-45* rescue at the restrictive temperature. Rescued animals were then crossed with strain DP246 carrying a balanced lethal *(st601)* allele of *unc-45*. F1 heteroallelic early adults *(r450ts/st601let)* were grown at the restrictive temperature overnight, dissected and placed onto 3% agarose pads; embryos remained in the uterus of dissected adults to protect the fragility of early embryos.

Two-hybrid screen

Full-length *unc-45* cDNA was amplified from pDP#WA036 by PCR using the primers 5′-TTTCCCGGGATGGTTGCTCGAGTACAG-ACT-3′ and 5′-CAACCCGGGTTCCTGAATGGTGCTCATTTG-3′. The PCR product was digested with *Sma*I and cloned into pGBDU-C1 (James et al., 1996), to give plasmid pDP#WA039. This was used as a bait to screen ~10^6 transformants of a *C. elegans* cDNA mixed stage expression library in the λACT-RB1 vector (kindly provided by Robert Barstead, Oklahoma Medical Research Foundation, Oklahoma City, OK). Three strong positive clones and several weaker ones were identified. These were re-tested by purifying the plasmid DNAs and re-transforming them into yeast. As controls, the bait plasmid pSE1112 (Durfee et al., 1993), which encodes the SNF1 fusion protein, and FEM-2 (Pilgrim et al., 1995) were tested. After sequencing the cDNA fragments and re-cloning into pGAD vectors, their interactions with UNC-45 were confirmed a third time. The two positive cDNAs that will be discussed are a 1.6 kbp fragment of *nmy-2* and a 420 bp fragment of *hum-2*. A third strong positive, *Ce*UFD2, required the TPR domain for an interaction and was therefore not examined further.

To test different domains of UNC-45 with these positives, *unc-45* subclones were constructed. A 2.6 kbp *unc*-45 cDNA fragment was amplified by PCR from pDP#WA039 using primers 5′-CAACC-CGGGTTCCTGAATGGTGCTCATTTG-3′ at the 3′ end of *unc-45* and 5′-GATCCCGGGATTGTTGAAGTTCTTCAG-3′ at the 5′ end of exon 4. It was cloned into pGBDU-C1, resulting in plasmid pDP#WA040, which encodes a truncated UNC-45 protein lacking the TPR domain (the first 112 amino acids at the N-terminus).

Quantification of the yeast two-hybrid interactions was performed using the Pierce quantitative β-galactosidase assay kit according to kit specifications.

RNA interference (RNAi)

RNA mediated interference has been shown to be a useful tool for phenocopying a null mutation in many genes (Fire et al., 1998; Montgomery et al., 1998; Tabara et al., 1998). The partial *nmy-2* cDNA was re-cloned into pBluescript KS$^-$ (Stratagene). Double-stranded RNA was prepared using a MEGAscript T7 in vitro transcription kit (Ambion) using the primers 5′-AGCTCGGTA-ATACGACTCACTATAGGGAAC-3′ and 5′-CCAGTGAATTGTA-ATACGACTCACTAT-3′. After microinjection of the RNA into the gonad of adult hermaphrodites, the same phenotype for *nmy-2* was observed in the progeny as previously described (Guo and Kemphues, 1996). To generate RNAi-treated worms for immunostaining, a soaking method was used (Tabara et al., 1998; Timmons and Fire, 1998). Briefly, 1 μl Lipofectin (Gibco-BRL) and 4 μl dsRNA prepared as above were mixed and incubated at room temperature for 15 minutes. 15-20 L4-stage hermaphrodites were added to the dsRNA-liposome mixture, and incubated for 10-24 hours at room temperature. Following incubation, the worms were transferred to an agar plate with *E. coli* (strain OP50) and resulting progeny were examined as described below.

Immunofluorescence microscopy

Embryos from either wild-type or *nmy-2* RNAi-treated hermaphrodites were fixed as described (Miller and Shakes, 1995) using methanol/acetone followed by air-drying. To stain the adult gonads, the worms were placed on a slide in M9 buffer and cut beneath the pharynx to release the intact gonads from the worm bodies. These were stained using the same protocol. Mouse monoclonal antibody 5-8 against MHC B (kindly provided by David Miller III, Vanderbilt University, Nashville, TN) and mouse monoclonal MH27 (provided by Joel Rothman, University of California Santa Barbara, CA) (Francis and Waterston, 1985), were used at 1:1000 dilutions. Rabbit polyclonal antisera against NMY-2 (a gift from Ken Kemphues, Cornell University, Ithaca, NY) were used at 1:200 dilution and rabbit polyclonal antisera 7N5 against UNC-45 (Ao and Pilgrim, 2000) were used at 1:500-1000 dilution. Pre-immune serum from the same rabbit (7N5) was used as an UNC-45 negative control. The secondary antisera (Sigma) were FITC-labeled anti-rabbit or TRITC-labeled anti-mouse (both at 1:1000 dilution). For the UNC-45 and NMY-2 colocalization experiment, UNC-45 antibody 7N5 was directly labeled with FITC as described (Harlow and Lane, 1999). The FITC-labeled antisera were used at a 1:50 dilution. DAPI was used at 1 µl/ml in the mounting media. The immunofluorescence images were taken using an Axioskop (Carl Zeiss) or by confocal microscopy (Molecular Dynamics 2001) and processed using Photoshop 5.0 (Adobe).

Results

UNC-45 protein is contributed to the embryo through the maternal germline

The maternal effect seen with strong alleles of *unc-45* predicts that *unc-45* mRNA or protein (or both) are contributed though the maternal germline. In situ hybridization shows that *unc-45* mRNA is enriched in the gonad of adult worms as well as being quite strong in two- and four-cell embryos (Dr Y. Kohara, online database at http://nematode.lab.nig.ac.jp/dbest/srchbyclone.html, EST yk44f2). In older embryos, the mRNA is localized in the presumptive muscle quadrants and the protein is also detectable in these tissues. Fig. 1 shows UNC-45 staining in the hermaphrodite gonad distal arm (Fig. 1A) and in all cells of the early embryo until the stage at which morphogenesis begins (Fig. 1C-E). Although staining is visible throughout the cell, it is especially apparent at the earlier stages

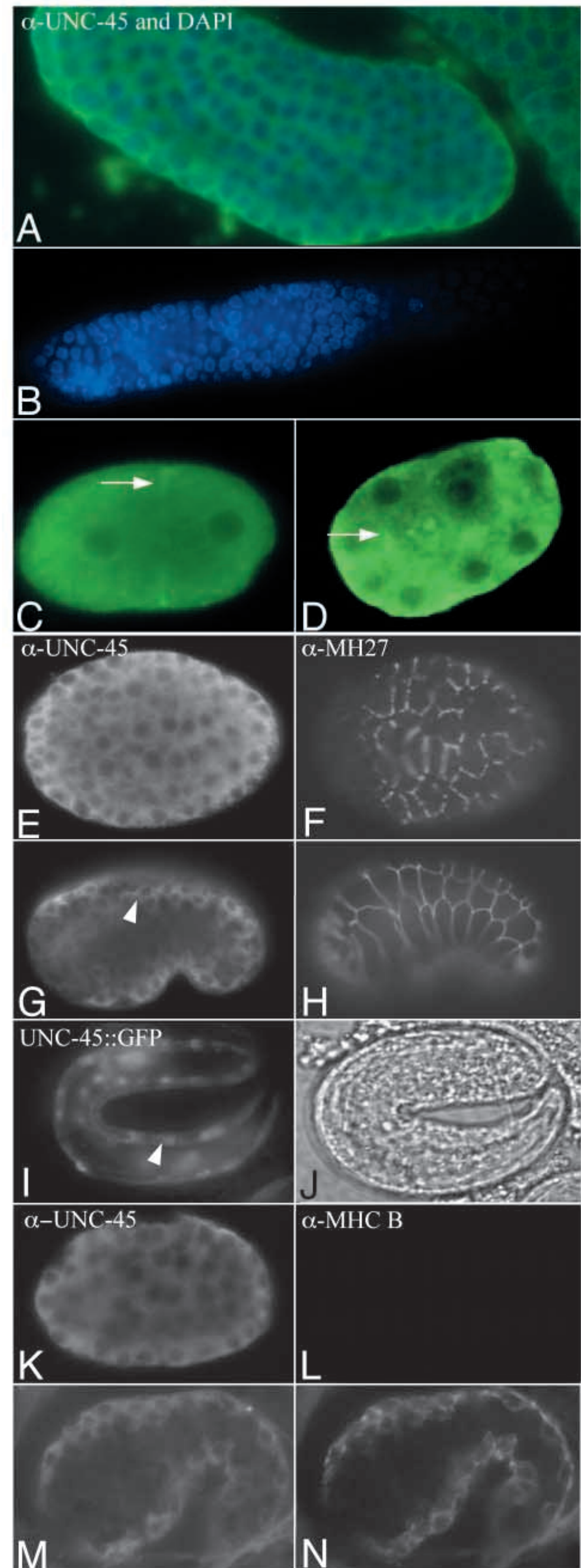

Fig. 1. UNC-45 expression in the nematode *Caenorhabditis elegans* adult germline and early embryo. (A) A hermaphrodite gonad doubly stained with anti-UNC-45 antibody (green) and DAPI (blue), which stains the cell nuclei. (B) Distal part of hermaphrodite gonad doubly stained with pre-immune serum and DAPI as controls. (C,D) Two-cell (C) and ~16-cell (D) embryos stained with anti-UNC-45. Arrows indicate concentration of UNC-45 at cell boundaries. In panels E-H, embryos are doubly stained for UNC-45 (E,G) and monoclonal MH27 (F,H), which recognizes an epitope associated with adherent junctions at the boundaries of hypodermal cells (Francis and Waterston, 1985; Hresko et al., 1994). As the embryo begins to elongate, the UNC-45 staining is progressively restricted to the presumptive muscle cells (G, arrowhead). (I,J) GFP and DIC images of a two-fold embryo transgenic for an UNC-45::GFP fusion protein, showing expression in the body wall muscles (arrowhead). (K-N) UNC-45 staining is detectable throughout embryogenesis whereas MHC-B staining is only detectable in the developing body wall muscles. K and M are stained with anti-UNC-45, L and N with anti-MHC B (5-8). (K,L) The same embryo at about 250 minutes after fertilization. (M,N) The same embryo at the 1.5-fold stage of elongation. The images of L and N were exposed for the same times, serving as controls to show that although MHC B does not show staining in the early embryos, UNC-45 does.

and appears to be concentrated at the cell cortex (Fig. 1C,D). As transcription of zygotic genes does not usually begin until several cell cycles have passed (Seydoux et al., 1996; Seydoux and Dunn, 1997), this is consistent with maternal contribution of UNC-45 protein and mRNA to the oocyte, and persistence at least until gastrulation. We can indirectly determine the time of onset of zygotic *unc-45* transcription by examining reporter genes. Transgenic arrays are often silenced in the *C. elegans* germline (Kelly et al., 1997); thus, reporter gene expression from such an array usually only represents the zygotic somatic component. We have seen GFP expression only in the muscle cells and not in either the germline or oocytes from *unc-45::GFP* reporter constructs (Venolia et al., 1999) (Fig. 1I) and expression is detected prior to appearance of MHC B at the bean stage of embryogenesis (data not shown). Consistent with this, UNC-45 protein is detected by immunostaining prior to detection of MHC B (Fig. 1K,L). At 270 minutes after fertilization, maternal UNC-45 is evenly distributed in all cells (Fig. 1E) but after 300 minutes, staining is more concentrated in muscle cells (the lateral bands of muscle cells are apparent in Fig. 1G). At the 1.5-fold stage of elongation, the zygotic expression of UNC-45 is clearly colocalized with MHC B in muscle cells (Fig. 1M,N). Other muscle structural proteins are first detectable in the same regions at about this time (Epstein et al., 1993; Hresko et al., 1994; Moerman and Fire, 1997). Although it is not possible to exclude the possibility that maternal UNC-45 becomes concentrated in the muscle cells at later stages, a simpler explanation is that maternal UNC-45 protein persists in all cells of the embryo until gastrulation. As the embryo begins to elongate, maternal protein staining disappears and zygotic UNC-45 is synthesized in a muscle-cell specific manner.

UNC-45 is required for proper cytokinesis during embryogenesis

As zygotic expression of *unc-45* is sufficient for normal muscle structure (Venolia and Waterston, 1990), what is the role of maternal UNC-45? If UNC-45 has a role in the early embryo, that role would only have become apparent in embryos sufficiently depleted of UNC-45; that is, in those embryos resulting from a mother homozygous for an *unc-45* null allele. As the strongest alleles of *unc-45* are zygotic lethal at the two-fold stage of embryogenesis (Pat phenotype), these adults do not normally exist and such a function would not have been apparent from previous analysis (Venolia and Waterston, 1990).

To separate genetically the possible roles of UNC-45 in the adult muscles from the germline and early embryo, we relied on both the muscle rescuing ability and the apparent lack of germline expression of the pDP#WA036 transgenic construct as described above. Transgenic animals homozygous for the temperature-sensitive (ts) *unc-45* allele *r450* are viable and fertile at the restrictive temperature (see Venolia et al., 1999). Transgenic animals homozygous for the *unc-45* lethal allele *st601* were somatically rescued for movement, but sterile, with a visibly abnormal germline (not shown). No GFP expression was seen in the germline of the transgenic lines, but we assume that the *r450* ts allele at the restrictive temperature provides at least some germline function sufficient for fertility, consistent with the provision of at least some somatic muscle function in *r450* ts homozygotes (Ao and Pilgrim, 2000). This leads us to postulate that UNC-45 has an essential germline function that is lacking in the transgenic *unc-45(st601)* homozygous animals as well as in RNAi treated animals (below).

Neither the *r450* ts allele nor the *st601* lethal alleles show any dominant effects on early embryogenesis (not shown). We next examined animals heteroallelic for the ts and lethal alleles, carrying the pDP#WA036 transgene. The aim was to produce viable and fertile adult animals, but with a reduced UNC-45 function provided to the embryos. *r450/st601* genotype in the absence of the transgene is lethal at the twofold stage. At the restrictive temperature, transgenic animals showed near-normal movement; the germline abnormalities seen in animals homozygous for the lethal allele were ameliorated and fertilization took place, but no viable progeny resulted. Instead, the temperature-depleted embryos produced from these somatically rescued heteroallelic adults showed defects much earlier than the previously reported 'Pat' phenotype. Embryos depleted for maternal UNC-45 exhibit several defects, most obviously frequent failure to complete cytokinesis, whereas nuclear division proceeds, often resulting in apparently polyploid daughter cells (Fig. 2). The embryos depleted for UNC-45 may have initially established A/P polarity (based on the attempt to form cleavage furrows in asymmetrical positions along the embryo), but the most severely affected embryos failed to complete a single cytokinesis. Typically, embryos attempt cytokinesis with varying degrees of furrow ingression; few if any successfully complete cytokinesis on the first attempt (0/8 embryos examined by time lapse). Following cytokinetic failure, the furrows subsequently regress. The embryo repeatedly attempts cytokinesis each time the nuclei divide (over at least one hour of observation) and on these attempts, the furrow would only occasionally complete (3/8 embryos examined). Temperature-depleted embryos that successfully complete one cytokinetic cleavage invariably fail in subsequent rounds, while nuclear division appears to continue. No viable embryos have been recovered out of the hundreds examined. This effect is due to the presence of the *st601* nonsense allele in the mother, because embryos produced at the restrictive temperature by homozygous *r450/r450* adults do not exhibit cytokinetic defects, and do not arrest at the twofold stage.

We attempted to determine the role of UNC-45 in the early embryo using RNA-mediated interference. As reported previously (Venolia et al., 1999), embryos resulting from broods laid by adults 7-8 hours after injection of a dsRNA corresponding to part of the *unc-45* cDNA showed phenotypes consistent with arrest at the two fold stage of embryogenesis. Adults treated with RNAi for longer times inevitably become sterile. Sterility caused by *unc-45* knockdown in a subset of animals following RNAi treatment has also recently been reported by others, who examined *unc-45* as part of a genome-wide screen (Kamath et al., 2003). We re-examined the broods of *unc-45* RNAi injected animals, concentrating our attention on the last eggs laid before sterility results. We were able to identify embryos from each brood that showed very little furrow invagination and a more severe defect than the temperature-depleted animals, consistent with that seen for the temperature depletion of *unc-45*. Therefore, maternally provided UNC-45 appears to be essential and has a previously unrecognized role in embryonic cytokinesis.

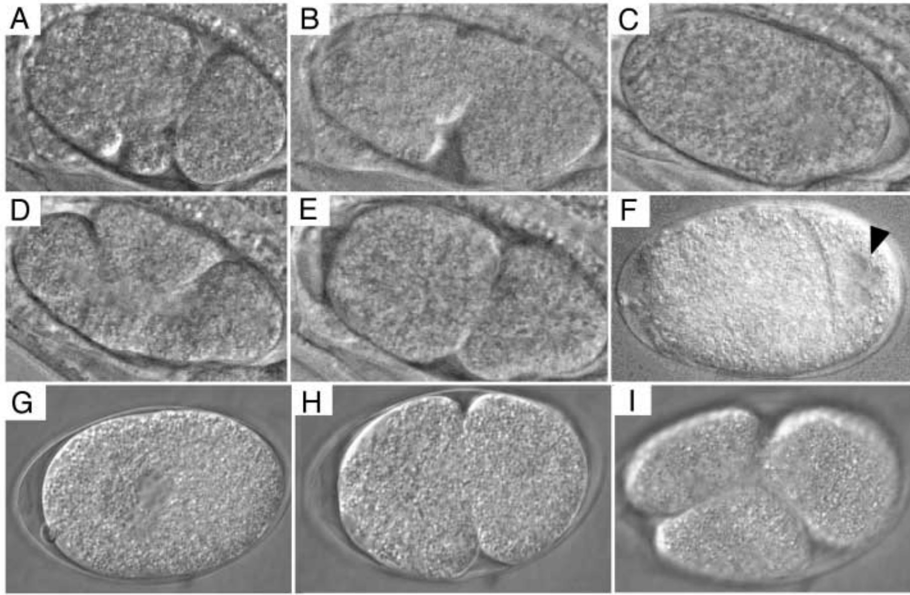

Fig. 2. Cytokinesis defects in *C. elegans* embryos derived from mothers of the genotype *unc-45(st601let) / unc-45(r450ts)* carrying an extrachromosomal array with the wild-type *unc-45* cDNA fused to GFP, raised at 25°C. (A-E) Time course shows the same embryo photographed at different times. Complete cytokinesis was not observed, although many attempts were made. Times are relative to the beginning of observation: (A) 0 minutes; (B) 15 minutes; (C) 25 minutes; (D) 40 minutes; (E) 70 minutes. (F) Embryo from a different mother of the same genotype. Note the multiple nuclei in one cell (arrowhead). (G-I) A wild-type embryo is normally able to complete at least two cell divisions in a similar time. (G) 0 minutes, pronuclear meeting; (H) 4 minutes, first cleavage; (I) 14 minutes, second cleavage. Embryos were observed under differential interference contrast optics.

UNC-45 interacts with non-muscle myosins

UNC-45 colocalizes with MHC B in the body wall muscle, and the yeast homologue Rng3 interacts with myosin in vivo. Therefore, we attempted to identify other myosins that may interact with UNC-45 in vivo using the yeast two-hybrid method (Fields and Sternglanz, 1994; Phizicky and Fields, 1995; Gietz and Schiestl, 1995; James et al., 1996) to screen a *C. elegans* cDNA library using the full-length *unc-45* cDNA as bait. Several weak and three strong positives were identified from 10^6 transformants examined. Purifying the plasmid DNAs and re-transforming them into yeast confirmed the positive interactions of UNC-45 with the preys. As a control, bait plasmids encoding the SNF1 fusion protein (Durfee et al., 1993) and the FEM-2 protein phosphatase (Pilgrim et al., 1995) were tested. SNF1 displayed a positive interaction with SNF4 protein in the yeast assays as expected, but neither SNF1 nor FEM-2 interacted with the putative UNC-45-dependent positives as judged by lack of growth on appropriate selective media. After sequencing the cDNA fragments and cloning into a different ‘prey’ vector backbone (pGAD) (James et al., 1996), their interactions with UNC-45 were reassayed in yeast, and the same results were obtained.

Of the strong positives (Fig. 3), two encode non-muscle myosin proteins: one is a fragment of *nmy-2*, and the other a fragment of *hum-2*, a class V myosin (Baker and Titus, 1997); in both cases the fragments correlate to regions within the head domain of the myosins. Class V myosins may act as vesicular motors (Titus, 1997). The third positive was identified as UFD2, however further analysis was not pursued. UNC-45 has two regions of sequence similarity to other proteins: the TPR domain at the amino terminus, and the UCS domain at the carboxyl terminus. The UCS family members (CRO1, She4p and Rng3p) all lack the TPR domain, suggesting that a specific myosin-binding activity may be reflected in the conserved similarity, and the portion of UNC-45 C-terminal to the TPR domain has been reported to bind myosin in vitro (Barral et al., 2002). To address this with respect to HUM-2 and NMY-2, we tested a subclone of UNC-45 containing solely the TPR domain (amino acids 1-112). Neither HUM-2 nor NMY-2 showed significant interaction with bait containing just the TPR domain of UNC-45. Therefore, the myosin-binding domain lies within the larger fragment of UNC-45 containing the UCS domain.

The isolated cDNA fragments of the two myosin molecules encode only a small part of the entire proteins, which are nearly 2000 amino acids in length. A 530 amino acid fragment of the NMY-2 head domain (amino acids 469-999) is sufficient for the two-hybrid interaction with UNC-45 (Fig. 3). The fragment of *hum-2* corresponds to amino acids 540-680 in the head domain that ends downstream of the ATP binding site and begins about 20 amino acids upstream of the actin-binding site. Thus, the fragments of myosin isolated in each of the two cases overlap (Fig. 3). This small region also overlaps with a region of MHC B that is sufficient to localize UNC-45 to thick filaments in vivo, using MHC A/B chimeric molecules (Hoppe and Waterston, 1996) (P. Hoppe, W.A. and D.P., unpublished).

In order to estimate the strength of the yeast two-hybrid interactions a quantitative β-galactosidase activity assay was performed. There is no significant difference in the level of reporter gene activity with NMY-2 compared to HUM-2 (Fig. 3B) when full-length UNC-45 is expressed as bait. However, the TPR domain alone shows minimal interaction with either myosin as shown by the lack of growth and low β-gal activity. Therefore, we have shown that NMY-2 interacts specifically with UNC-45.

UNC-45 colocalizes with NMY-2 in the early embryo

For the two-hybrid interaction to be meaningful, it is necessary to show that the two proteins are at least expressed in the same cells at the same time. Although the expression pattern of HUM-2 has not been reported, a strain homozygous for a putative null allele (*ok596*) is viable, fertile and moves normally, suggesting that the embryonic lethality of *unc-45* is unlikely to result from effects on HUM-2. Both maternal UNC-45 and NMY-2 proteins are present in the early embryo; NMY-2 was initially identified as a non-muscle myosin II heavy chain that interacts with the C-terminus of PAR-1, a putative kinase

(Guo and Kemphues, 1996). NMY-2 is necessary in the embryo for normal asymmetric localization of PAR-1, -2 and -3 (Guo and Kemphues, 1996). Furthermore, NMY-2 is localized at the embryonic cleavage furrow of the two-cell embryo and required for polarized cytoplasmic flow and cytokinesis (Guo and Kemphues, 1996; Shelton et al., 1999). A non-muscle myosin regulatory light chain has also been implicated in this process (Shelton et al., 1999). The staining seen for UNC-45 (Fig. 1C,D) matches the staining reported for NMY-2 (Schumacher et al., 1998; Shelton et al., 1999), so we examined the localization of both proteins in the same embryos. We examined embryos labeled with NMY-2 and UNC-45 antibodies under regular and confocal microscopy. Fig. 4A-F shows images of two-cell and ~20-cell stages of wild-type embryos, demonstrating that NMY-2 and UNC-45 are indeed concentrated at the cell cortex, and the staining patterns are largely coincident (although there are slight differences in intensity). Cortex staining for UNC-45 is apparent both where a fluorescent secondary antibody is used, and where UNC-45 antisera is directly labeled with a fluorescent marker (data not shown). Therefore, the two proteins colocalize in vivo, supporting the two-hybrid evidence that they may physically interact.

It remains to be determined whether this interaction is productive in vivo. However, we have shown that removal of MHC B protein from the thick filament results in lack of subcellular localization of UNC-45 staining (Ao and Pilgrim, 2000), whereas wild-type UNC-45 is not necessary for at least partial localization of MHC B to the thick filament. RNAi directed against *nmy-2* results in embryos defective in asymmetric cell division and cytokinesis (Guo and Kemphues, 1996; Shelton et al., 1999). In Fig. 4G,H, the UNC-45 staining at the cortex of the cell is disrupted in *nmy-2(RNAi)*-treated embryos, indicating that the localization of UNC-45 at the cleavage furrow is dependent on NMY-2. Thus, UNC-45 has an embryonic localization that is NMY-2 dependent, and

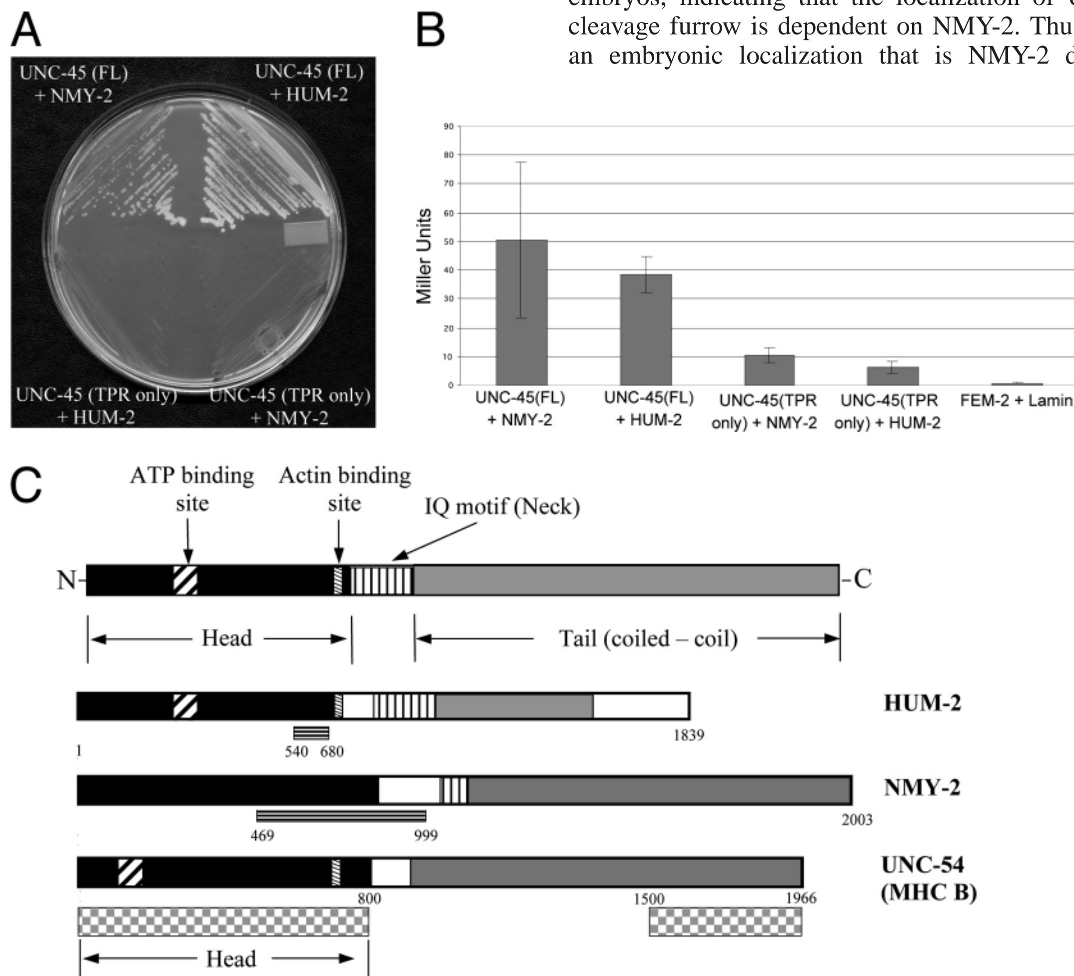

Fig. 3. Characterization of isolated clones from a yeast two-hybrid screening of a *C. elegans* cDNA library with *unc-45* cDNA. (A) Two of the clones identified from yeast two-hybrid screens. UNC-45 from a full-length cDNA (pDP#WA036) interacts with the products of fragments of two different myosin cDNAs, NMY-2 and HUM-2. The TPR domain alone was tested and showed no interaction with either NMY-2 or HUM-2. (B) Quantitative β-gal assay for the yeast two-hybrid clones. (C) Diagram of a conventional myosin II molecule, showing the ATP and actin binding sites in the head domain, IQ motif (light chain and calmodulin binding sequence) in the neck domain and the coiled-coil tail domain. Schematic diagram of MHC B and two myosins identified from the yeast two-hybrid screens that interact with UNC-45. HUM-2 is a type V myosin and the identified cDNA fragment encodes a 140 amino acid segment located in the head domain (striped shading). NMY-2 is a non-muscle type II myosin and a region of 530 amino acids (striped shading) located mostly in the head domain interacts with UNC-45. Note that these two identified regions overlap in the highly conserved head domain as indicated. In UNC-54, the portion of the molecule that is sufficient to localize UNC-45 to the body wall muscle thick filaments is indicated by checked shading. Detailed domain analysis of NMY-2 is not available.

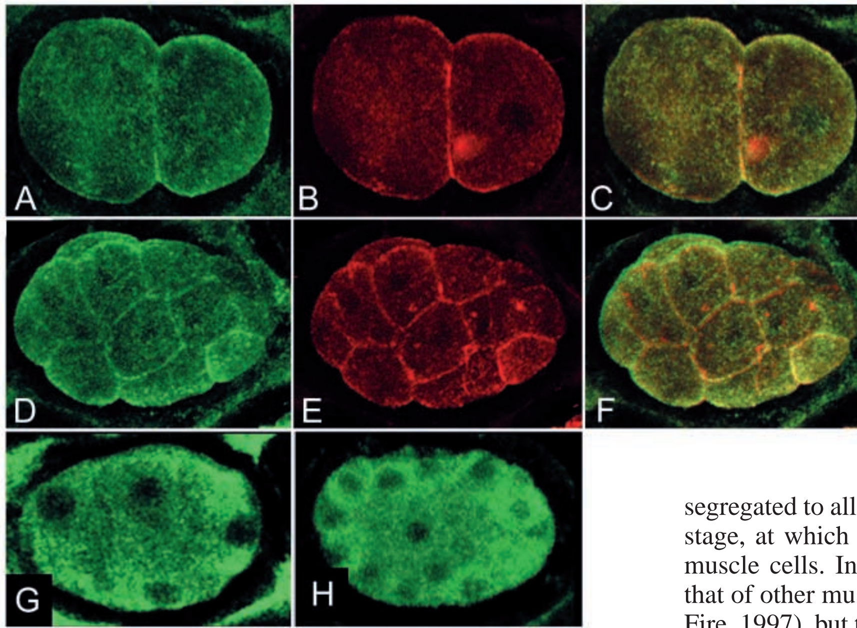

Fig. 4. Immunolocalization of UNC-45 and NMY-2 in wild-type and *nmy-2*(RNAi) embryos. (A-F) Wild-type embryos of increasing age stained with anti-UNC-45 (green) and anti-NMY-2 (red) to show the concentration of UNC-45 and NMY-2 at the cell boundaries. (A-C) Two-cell embryo with the merged image showing colocalization of UNC-45 and NMY-2. (D-F) ~20-cell stage embryo with merged image. Images G and H show embryos treated with RNA-mediated interference (RNAi) against NMY-2, stained with anti-UNC-45 showing that the concentration of UNC-45 at the cell boundaries is disrupted when NMY-2 is knocked down.

NMY-2 is similar to MHC B, in that removal of the protein results in a change in subcellular localization of UNC-45. We examined NMY-2 immunostaining in a temperature depleted *unc-45* background. The localization of NMY-2 remained cortical and no differences were detected from wild-type control embryos (data not shown). This result is expected from the minimal cytokinetic furrow activity indicating residual function for the UNC-45 maternal contribution of the *r450* allele.

Discussion

Here we provide evidence for an UNC-45 interaction with NMY-2, a non-muscle myosin, and a biological role for the UNC-45/NMY-2 interaction during embryonic cytokinesis. Previously, the evidence that UNC-45 or its mRNA is contributed maternally was indirect, as the lethal alleles of *unc-45* show maternal effects (Venolia and Waterston, 1990). All reported phenotypes of *unc-45* mutants could be zygotically rescued, although the precise nature of the defect that leads to the lethality has not been adequately explained (Venolia and Waterston, 1990). Reporter transgenes in *C. elegans* are poorly expressed in the germline (Seydoux et al., 1996; Seydoux and Dunn, 1997; Kelly and Fire, 1998), and any germline expression or maternal contribution of *unc-45* product would have been overlooked by the use of a GFP reporter. In situ hybridization of whole animals using *unc-45* cDNA as a probe suggests that *unc-45* transcripts are widespread in the early embryo as well as being strongly expressed in the adult hermaphrodite germline. There has not previously been an explanation for the detection of widespread early embryonic *unc-45* transcripts, as body wall muscle and pharynx muscle components are synthesized from zygotically transcribed genes. We show here that both *unc-45* mRNA and the protein are maternally provided. Since the antisera detects both maternal and zygotic UNC-45, the simplest explanation for the differences that we see between the immunolocalization and the reporter gene expression is that maternal UNC-45 is segregated to all cells in the embryo, and persists until the bean stage, at which point zygotic UNC-45 is synthesized only in muscle cells. In this way, zygotic UNC-45 synthesis mirrors that of other muscle thick filament components (Moerman and Fire, 1997), but the existence of a maternal contribution among this set of genes would be unique.

Why is UNC-45 mRNA and protein provided maternally to the embryo if it is not required until later in zygotic development? The localization of UNC-45 staining to the cell cortex is not unique, as several maternally contributed components show cortical localization in the two-cell embryo (Rose and Kemphues, 1998). Non-muscle myosin II NMY-2 (Guo and Kemphues, 1996) is also enriched in the germline cells of the adult and at the cortex of early embryos. The observations that UNC-45 and NMY-2 colocalize in the embryo, that UNC-45 interacts with a fragment of NMY-2 in the yeast two-hybrid assay, and that UNC-45 localization in the embryo is disrupted if NMY-2 is removed using RNAi suggest a specific function for UNC-45 at this stage. We have now been able to define a precise role for maternally provided UNC-45 during embryonic cytokinesis. NMY-2 has been shown to be required for cytokinesis (Guo and Kemphues, 1996) and a non-muscle myosin regulatory light chain, MLC-4, is also involved in this process (Shelton et al., 1999). Thus, this is the second instance of UNC-45 subcellular localization being dependent on a type II myosin molecule, potentially acting in a similar manner to aid in the assembly or stability of a myosin molecule during cytokinesis.

The colocalization of UNC-45 with body wall muscle thick filaments in an MHC B dependent manner suggested, but did not prove, that the interaction between UNC-45 and MHC B was direct. Our observation from this work strengthens that inference, as do in vitro studies (Barral et al., 2002). As mentioned above, both UNC-45 and NMY-2 are expressed in the germline cells of the gonad as well as the pre-morphogenesis embryos, and the localization of maternal UNC-45 at the cell boundaries is dependent on the presence of NMY-2. In addition, UNC-45 and NMY-2 depletion both affect the same process of embryonic cytokinesis, further strengthening the idea that the UNC-45/NMY-2 interaction is biologically relevant. We found that polarity is established and maintained in UNC-45 heteroallelic temperature-depleted embryos indicated by an asymmetrically placed cleavage furrow suggesting either that the residual function of UNC-45

is sufficient to allow for polarity, or that NMY-2 does not require UNC-45 for establishment of the anterior-posterior axis. Consistent with residual UNC-45 function, no defect in NMY-2 localization was observed for the temperature-depleted embryos and NMY-2 remained cortical.

The interaction of UNC-45 with HUM-2 in the two-hybrid screen, although intriguing, has not been confirmed using an independent assay. In addition, until it is shown that the expression patterns of UNC-45 and HUM-2 overlap, we can only state that the interaction is possible in vivo. This may be somewhat surprising as MHC B and NMY-2 are type II myosins, whereas HUM-2 is Type V (Baker and Titus, 1997). These non-muscle myosin classes are largely differentiated based on similarity of the sequences of their head domains as well as the length of the rod domains (Titus, 1997; Baker and Titus, 1997). It has been reported recently that a member of the UCS family of proteins in budding yeast, She4p, can interact with a type V myosin as well as a type I myosin (Wesche et al., 2003). However, there is striking sequence similarity in the regions of the head domain that is common to all three myosins. We assume that the biochemical role that UNC-45 has in its interaction with MHC B in the body wall muscle is related to its interaction with NMY-2, and perhaps to HUM-2. Although UNC-45 has demonstrated chaperone activity in vitro (Barral et al., 2002), no chaperone activity has been shown in vivo although such an activity is consistent with the mutant phenotype. As the role of non-muscle myosin II, particularly in cytokinesis, is a dynamic one where there is constant remodeling of the cytoskeleton, it may certainly involve chaperones and associated factors necessary for targeting and folding of myosin molecules (Bresnick, 1999; Young et al., 2003). Rng3, which has sequence similarity to UNC-45 in fission yeast is also involved in cytokinesis and has been shown to interact genetically with a type II myosin (Wong et al., 2000). Therefore, this may be an evolutionarily conserved process.

As UNC-45 clearly acts through myosin in other tissues, it seems most likely that its role in the embryo is mediated (at least in part) through NMY-2. Therefore, unless there is evidence for a completely independent role for UNC-45, the simplest explanation is that UNC-45 is provided to assist NMY-2 in proper assembly into some higher order complex, to stabilize assembled myosin molecules and/or to localize NMY-2 to the proper subcellular location. Indeed, such a role may not be limited to embryos as a cytokinetic mechanism is necessary for proper oogenesis; transgenic *unc-45* null hermaphrodites are sterile because of a failure to produce oocytes (T.K. and D.P., unpublished). Similarly, the F1 progeny of *nmy-2* RNAi-treated animals that escape the embryonic lethality often develop to become sterile adults (Kamath et al., 2003) suggesting that NMY-2 may also be required in germline development, perhaps in a mechanism that also involves UNC-45. The UNC-45 homologue CRO1 is necessary for transition from syncytial to cellular growth in *Podospora anserina* (Berteaux-Lecellier et al., 1998), a process analogous to germ cell development in the *C. elegans* gonad (Schedl, 1997) and *C. elegans* UNC-45 may play a similar role in many different tissues.

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