Two mammalian UNC-45 isoforms are related to distinct cytoskeletal and muscle-specific functions

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Summary

Previous studies have shown that the UNC-45 protein of *C. elegans* is required for normal thick filament assembly, binds Hsp90 and the myosin head, and shows molecular chaperone activity. We report here that mice and humans each have two genes that are located on different chromosomes, encode distinct UNC-45-like protein isoforms, and are expressed either in multiple tissues or only in cardiac and skeletal muscles. Their expression is regulated during muscle differentiation in vitro, with the striated muscle isoform mRNA appearing during myoblast fusion. Antisense experiments in C2C12 skeletal myogenic cells demonstrate that decreasing the general cell isoform mRNA reduces proliferation and fusion, while decreasing the striated muscle isoform mRNA affects fusion and sarcomere organization. These results suggest that the general cell UNC-45 isoform may have primarily cytoskeletal functions and that the striated muscle UNC-45 isoform may be restricted to roles in muscle-specific differentiation.

Key words: UNC-45, Muscle differentiation, Proliferation, Chaperone, Myosin

Introduction

Myosins are key components in many cell processes, including muscle contraction, cell division and membrane trafficking. Consistent with these multiple roles, myosins comprise a superfamily of at least eighteen major classes (Berg et al., 2001; Sellers, 2000). The human genome contains an estimated forty genes encoding myosin-type motors (Berg et al., 2001). Conventional myosins of the myosin II family are molecules of >500 kDa that assemble through their long α-helical coiled-coil domains. These myosins are major components of muscle thick filaments and are the motors of other actin-based contractile assemblies. All other myosin motors constitute the multiple classes of unconventional myosins. These myosins do not assemble into filaments because they lack the specific coiled-coil domains. Unconventional myosins are involved in a variety of actin-based processes, including actin filament formation, cell contraction, endocytosis and phagocytosis, and trafficking of membrane organelles (Reck-Peterson et al., 2000; Wu et al., 2000). It has been estimated that the typical mammalian non-muscle cell contains about ten unconventional myosins in addition to a conventional myosin (Bement et al., 1994).

Myosin heads irreversibly aggregate in bacterial expression systems and do not show motor function, suggesting that necessary factors are missing [(McNally et al., 1988); see (Srikakulam and Winkelmann, 1999) for discussion]. The heads of muscle and non-muscle myosin differ in their ability to fold properly upon expression in non-muscle (insect) cells. Heads of cytoskeletal and smooth muscle myosins II, V and VI expressed as subfragment 1 or heavy meromyosin regions are soluble, functional proteins (Sweeney et al., 1998; Wang et al., 2000; Wells et al., 1999), however cardiac (sarcomeric) myosin heads are not (J. R. Sellers and H. L. Sweeney, personal communication). These results suggest that non-muscle eukaryotic cells contain factors necessary for folding the non-muscle myosin motors (see Hutagalung et al., 2002). Previous research suggests that molecular chaperones assist the folding of muscle myosin heads. The chaperonin containing TCP-1 (CCT, where TCP-1 is t complex polypeptide 1) (Kubota et al., 1995) associates with and enhances the folding of nascent skeletal muscle heavy meromyosin in reticulocyte lysates (Srikakulam and Winkelmann, 1999). In addition, cultured muscle (C2C12) cells but not epithelial cells contain factors that permit the proper folding of recombinant muscle myosin subfragment 1 (Chow et al., 2002). The *C. elegans* protein UNC-45 has been shown by genetic experiments to be necessary for thick filament assembly (Epstein and Thomson, 1974; Venolia and Waterston, 1990) and by biochemical experiments to be a molecular chaperone with activity for the myosin head (Barral et al., 2002).

C. elegans UNC-45 protein has an apparent molecular mass of 107 kDa. It contains three amino-terminal tetratricopeptide repeats (TPR), a ~400 residue central region and a ~400 residue UNC-45/Cro1/She4p (UCS) domain (Barral et al., 1998; Venolia et al., 1999). The UNC-45 TPR domain binds the molecular chaperone Hsp90 in a stoichiometric manner (Barral et al., 2002). The remainder of the UNC-45 protein binds the myosin head and has chaperone activity on it (Barral et al., 2002). A temperature-sensitive mutation (*unc-45 e286*) in the UCS domain of UNC-45 reduced myosin accumulation and led to disordered assembly of the two myosin isoforms in body-

wall muscle thick filaments (Barral et al., 1998). The resulting thick filaments were unstable upon isolation. UNC-45 protein was localized to the A-bands of body-wall muscle by immunostaining, and to all muscle types of *C. elegans* by a GFP-reporter (Venolia et al., 1999; Ao and Pilgrim, 2000). UNC-45 also has a role in non-muscle cells, as shown by its immunodetection in cleavage furrows and its interaction with non-muscle myosin II and unconventional myosin V in two hybrid studies (W. Ao and D. Pilgrim, personal communication).

Yeast and other fungal proteins containing an UCS-domain were identified in several distinct mutant screens and show functional linkage to the cytoskeleton. CRO1 of *Podospora anserina* is necessary for multiple processes in cell division (Berteaux-Lecellier et al., 1998). She4p of *S. cerevisiae* is required for a normal actin-based cytoskeleton, endocytosis, and a myosin-V-based molecular transport process (Jansen et al., 1996; Wendland et al., 1996; Beach and Bloom, 2001). A more recently identified UCS-containing protein, *S. pombe* Rng3p, is crucial for cell shape, normal actin cytoskeleton, and contractile ring assembly (Balasubramanian et al., 1998). It is essential for assembly of the myosin II-containing progenitors of the contractile ring (Wong et al., 2002). Widespread defects in the cytoskeleton are found in null mutants of all three fungal proteins.

The present investigation was aimed at identifying mammalian UNC-45 gene products and gaining insight into their functions. While there is a single *C. elegans unc-45* gene, we found that humans and mice express two UNC-45-like gene products, one being present in all organs and the other highly expressed only in striated muscle. The expression of the general cell isoform decreased while the striated muscle isoform increased during muscle differentiation in vitro. Antisense experiments with the murine UNC-45 isoforms in the skeletal myogenic cell line C2C12 suggest that they have distinct roles.

Materials and Methods

Identification of mammalian UNC-45 cDNAs and cloning

Expressed sequence tagged (EST) fragments of mouse or human UNC-45 cDNAs were identified in the National Center for Biotechnology Information (NCBI) database by BLAST (Altschul et al., 1990; Altschul et al., 1997; Gish and States, 1993; Madden et al., 1996) searches using *C. elegans* UNC-45 [(Barral et al., 1998; Venolia et al., 1999) acc. no. AF034085 (DNA), acc. no. AF034085.1 (translated protein) Worm Base acc. no. NP_497205]. Longer mammalian cDNA fragments were constructed by Pfu polymerase (Stratagene, La Jolla, CA)-based polymerase chain reaction (PCR) across unknown sequences, using either a mouse 17 day embryo, human fetal brain or adult heart (Clontech, Palo Alto, CA) cDNA library as a template. Untagged full-length cDNAs were obtained with Herculase polymerase (Stratagene) and the following primers: human general cell UNC-45: 5′-ATG ACT GTG AGT GGT CCA GGG AC-3′ and 5′-TGA TAG CAG GCT CAC AGC TGG TGA GGC TGC-3′, mouse general cell UNC-45: 5′-ATG ACT GTG AGT GGC CCG AG-3′ and 5′-CTC TCC ATC CTG GTT GGG TTG-3′, mouse striated muscle UNC-45: 5′-GCT CAG AGC AGT GCC TAG GAG G-3′ and 5′-CAG TCT ACA GCC CGT TAT CTG GCC TGC-3′. cDNAs were cloned into the pCR-XL vector (Invitrogen, Carlsbad, CA). DNA sequencing in both directions (Lone Star Labs, Houston, TX) confirmed all cDNA clones. The Clustal W 1.8 multiple sequence alignment (Higgins et al., 1996) and Boxshade 3.3.1 programs [(Smith et al., 1996) see http://searchlauncher.bcm.tmc.edu] were used to align protein sequences.

Radiation hybrid chromosomal mapping of the human general cell UNC-45 locus

PCR with total human genomic DNA and primers 5′-CCA AGG CTC ATG CAC ACG CTA CCT ATT GTG G-3′ and 5′-TGA TAG CAG GCT CAC AGC TGG TGA GGC TGC-3′ amplified only the 325 bp of the 3′ UTR of human general cell UNC-45. This primer pair was utilized at Research Genetics (Huntsville, AL) for dual mapping on the Stanford and G3 human/hamster hybrid panels (Cox et al., 1990).

Northern blotting

Total RNA was isolated from mouse tissues or C2C12 myogenic cells following Chomczynski and Sacci (Chomczynski and Sacci, 1987) or with TRIzol (Life Technologies, Rockville, MD), or was purchased (ovary, whole embryo) (Ambion, Austin, TX). Methods for the RNA gels and northern blotting were modified from Sambrook et al. (Sambrook et al., 1989). RNA molecular weight standards ranging from 0.24 to 9.46 kb (Life Technologies) were used to generate a linear plot of migration distance. Ten μg of each sample was separated on a 1.5% agarose-formaldehyde gel. Blots were pre-hybridized and hybridized in UltraHyb solution (Ambion) at 65°C. Two different general cell UNC-45 probes were used, one was 955 bp of cDNA from the UCS region, the product of primer set 5′-CTC GGC ATT GGT CAA TTG CAC CAA CAG C-3′ and 5′-GGA TCT CCA GGA CCT CAC TCT CCA TCA GGG-3′, while the other was 550 bp, half of which is 3′ UTR and was the product of primer set 5′-CTC ACC TCC ATG CGG CCA CAC-3′ and 5′-GAT GCT CCC AGC ATG TGA GGA TGC-3′. The striated muscle UNC-45 probe was 755 bp of cDNA, 350 of which was 3′ UTR, and was the product of primers 5′-TAC GGC AGG CAG CCA CCG AAT GCA TGT G-3′ and 5′-CAG TCT ACA GCC CGT TAT CTG GCC TGC-3′. Twenty-five ng of each probe was labeled with [^{32}P]-dCTP (Amersham, Piscataway, NJ) by the random nonomer method to specific activities of 5×10^8-1×10^9 cpm/mg, and used at 1×10^6 cpm/ml. Blots were washed at room temperature with 2× sodium citrate/sodium chloride [SSC (see Sambrook et al., 1989)], 0.1% sodium dodecyl sulfate (SDS) for 2 minutes, and then at 63°C with three 15 minutes washes with 0.1× SSC, 0.1% SDS and 1 wash with 0.1× SSC, 0.5% SDS. Internal standards were obtained by re-labeling with a probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Ambion) or, in the case of multiple tissues constitutively expressing varying levels of GAPDH mRNA, with an 18 S RNA probe. Blots were exposed to Kodak BioMax MS film with BioMax intensifying screens for 3 days (general cell UNC-45), 11 hours (striated muscle UNC-45) or 2 hours (18S RNA and GAPDH) at –80°C. Films were digitized by scanning, and Photoshop (Abobe Systems, Mountain View, CA) was used for sizing and labeling. Absolute and relative amounts of general cell and striated muscle UNC-45 mRNA were obtained from films with UN-SCAN-IT software (version 5.1, Silk Scientific, Orem, UT) providing total pixels per band, and resulting data were analyzed using the Excel program (Microsoft, Redmond, WA).

In situ hybridization

The mouse general cell and striated muscle UNC-45 cDNA templates were identical to those used for northern blotting. Single-stranded sense and anti-sense digoxygenin-labeled RNA probes were generated as directed by the manufacturer (Roche Molecular Biochemicals, Indianapolis, IN). Whole-mount in situ hybridization was performed on mouse embryos of different ages as described in Conlon and Rossant (Conlon and Rossant, 1992). Labeled embryos were placed on a layer of agarose and photographed through a dissecting microscope with Kodak Ektachrome 160 T slide film. Slides were

digitized via a Sprint Scan scanner (Polaroid, Wayland, MA) and images were adjusted for scaling and size with the Photoshop program.

C2C12 mouse skeletal myogenic cell culture

Mouse C2C12 skeletal muscle cells were obtained from the American Type Culture Collection (ATCC CRL-1772) (Yaffe and Saxel, 1977; Blau et al., 1983; Silberstein et al., 1986). Cells were expanded and passaged 3 times, and frozen aliquots were used for antisense experiments. Cells were propagated in a humidified incubator at 10% CO_2 in growth medium consisting of Dulbecco's Minimal Essential Medium containing 4.5 mg glucose/L, 110 mg sodium pyruvate/L and supplemented with 10% fetal bovine serum (FBS), 0.05 mg/ml gentamicin and a 1:400 dilution of Fungizone (all components from Invitrogen, Carlsbad, CA). Differentiation was induced by medium containing 2% horse serum instead of 10% FBS. Phase-contrast images were taken with a 10× lens on Kodak TriX Pan film at ASA 400. Negative film images were digitized by scanning and imported into Photoshop for contrast adjustment and cropping.

Antisense oligonucleotide experiments

Phosphorothioate 21-mer oligonucleotides (Sigma-Genosys, Woodlands, TX) designed to anneal at the start codon were used for the suppression of the mouse general cell UNC-45 (5′-GCC ACT CAC AGT CAT CAC GAA-3′) and striated muscle UNC-45 (5′-TTC AGC CTC TGC CAT AGT CTT-3′). The control oligonucleotide had a base composition similar to the UNC-45 oligonucleotides but randomized (avoiding tandem repeats) (5′-TAA GCA CTA GGA CAC CTC CAC-3′). Second-generation chimeric 18-mer oligonucleotides (trademark of Oligos Etc. Inc, Wilsonville, OR) were also used: general cell UNC-45 antisense: 5′-CGC ATT TGA ACA GCT CGT-3′, a control oligonucleotide that reversed the order of the previous bases (5′-TGC TCG ACA AGT TTA CGC-3′), and striated muscle UNC-45 antisense: 5′CCA TGA GGC TGC AGA TTC-3′. Oligonucleotides were resuspended in 10 mM Tris, pH 8.5 and lyophilized in aliquots. They were added daily, starting 1 hour after plating, to between 1.25 and 5 μM. Proliferating cells were treated for up to 5 days, while myotube-forming cells were treated for up to 9 days. Cell number was assessed by three methods: counting adherent cells in defined areas, counting trypsinized cells resuspended in trypan blue stain, and by the CyQUANT proliferation assay (Molecular Probes, Eugene, OR). For the latter, 96 well culture plates were seeded with 2,000 cells/well. Eight wells or triplicates of eight wells were used for each 3-day treatment. Frozen cells were lysed with the kit's lysis buffer supplemented with NaCl to 180 mM and EDTA to 1 mM, and 2 Kunitz units of RNase A/ml. After 1 hour at room temperature, one volume of GR dye solution was added to a final concentration of 2× dye, and the fluorescence resulting from GR dye binding to DNA (Jones et al., 2001) was read on a CytoFluor plate reader at 485 nm excitation and 530 nm emission, with 3 reads per scan and a gain of 50. A standard curve showing a linear relationship was obtained using 2, 4, 8, 12, and $16{\times}10^3$ C2C12 cells. Data was tabulated and averages±standard deviations were calculated for the general cell or striated muscle antisense data separately paired with the control data using the Excel program. The Student's *t*-test was applied using the two-tailed, equal variance parameters.

Immunofluorescence microscopy

Cells were grown on Aclar coverslips (SPI Supplies, West Chester, PA) coated with 25 μg/ml of mouse basement membrane laminin (Sigma, St Louis, MO). Coverslips were washed twice briefly with DMEM minus serum, and fixed with 100% methanol at –20°C for 30 minutes. Coverslips were air-dried and stored under desiccating conditions at –80°C. Cells were hydrated and further permeabilized in phosphate-buffered saline (0.14 M NaCl, 2.5 mM KCl, 8 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) with 0.05% Tween 20 (Sigma) (PBST), blocked with 5% normal goat serum in PBST, and stained with antibodies diluted into PBST for 1 hour at room temperature. Primary antibodies were mouse monoclonal IgG clone EA53 (Sigma) against sarcomeric α-actinin, diluted 1:400 and supernatant of mouse monoclonal IgG2b MF20 directed against fast skeletal muscle, diluted 1:8 [Developmental Studies Hybridoma Bank (Bader et al., 1982)]. Alexa 488- or Alexa 594-labeled secondary antibodies (Molecular Probes, Eugene, OR) were used at 1:500 dilutions. DNA was labeled with 0.1 μg/ml 4′-6-diamidino-2-phenylindole (DAPI; Sigma). Coverslips were rinsed with glass-distilled water and mounted with Fluoromount G (Southern Biotechnology Associates, Birmingham, AL). Microscopy was done using an Olympus BX 60 (Olympus America, Melville, NY) or a Zeiss Axioplan 2 (Carl Zeiss, Thornwood, NY) epifluorescence microscope. Fluorochrome emission was examined individually, photographed on Fujichrome Provia 1600 slide film and developed with E-6 push processing prior to digitization and importation into Photoshop. Digital images as jpeg or Zeiss vision files were taken from the Axioplan microscope using Zeiss Axiovision software.

Immunoblotting

C2C12 myotubes cultures treated for 8 days plus or minus 2.5 μM antisense oligonucleotides were rinsed several times with Hank's balanced salt solution. Cells within one well of a six-cell plate were lysed with 0.2 ml of 150 mM NaCl, 20 mM Tris pH 7.4, 2 mM EDTA, 2 mM adenosine triphosphate, 5 mM dithiothreitol, 1% Triton X-100, and Complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN) to 5× concentration. SDS was then added to 1%. Proteins separated on 7.5% polyacrylamide gels were transferred to Immobilon-NC filters in 20% methanol in Laemmli running buffer, at 80 volts for 15 hours in the cold. Use of Kaleidoscope molecular weight markers (BioRad, Richmond, CA) and subsequent Commassie Blue-staining of the gels confirmed transfer. Blots were blocked with 1% nonfat dry milk in 150 mM NaCl, 50 mM Tris pH 7.6, 0.05% Tween-20, reacted with 2 μg/ml MF20 monoclonal anti-skeletal muscle myosin antibody (Bader et al., 1982), and counter-stained with 1:1000 dilution of horse radish peroxidase-labeled anti-mouse IgG. SuperSignal enhanced chemiluminescence (Pierce, Rockford, IL) and exposure to X-ray film detected labeling.

Results

Vertebrates have two UNC-45 isoforms that are products of separate genes

cDNA sequences encoding human and mouse UNC-45 proteins were identified using BLAST (Altschul et al., 1990; Altschul et al., 1997; Gish and States, 1993; Madden et al., 1996) searches of EST databases with the *C. elegans* UNC-45 amino acid sequence as a query. Two different isoforms, a general cell (GC) and a striated muscle type (SM), of mouse and human UNC-45 exist, as shown by PCR bridging reactions that utilized fragments of mouse or human UNC-45-like coding sequences (Fig. 1). The names of these isoforms are based on the differential expression of their mRNAs.

Like *C. elegans* UNC-45, the predicted sequences for the mammalian proteins consist of three distinct regions: an amino-terminal triple TPR motif, a unique central region, and a C-terminal UCS domain. Although the two mammalian UNC-45 isoform proteins predicted from full-length cDNA sequences are each 31-32% identical and 53-54% similar to *C. elegans* UNC-45, they are quite different from one another. Overall, the mammalian GC and SM UNC-45 isoforms are

only 55-56% identical and 74% similar in amino acid sequence. The most similar isoforms are 94-95% identical and 96-98% similar between mouse and human. Blocks of up to 10 consecutive identical and up to 25 conserved amino acids are present throughout the isoform sequences.

As indicated in Fig. 1, residues associated with dysfunctional mutations in *C. elegans unc-45* (red asterisks: in order, with residue number referring to that protein, *unc-45 b131* (G427E), *su2002* (L559S), *m94/r450* (E781K) and *e286* (L822F) or *S. pombe rng3* (blue asterisks: *rng3*-A3 (L483P) and *rng3*-65 (G688E) are identical or conserved in the mammalian UNC-45 proteins (Barral et al., 1998; Wong et al., 2000). The phenylalanyl and lysyl residues identified at the chymotrypsin and the trypsin cleavage sites, respectively, in *C. elegans* UNC-45 (J.M.B. and H.F.E., unpublished) are also closely conserved in the predicted mammalian proteins (indicated by blue and red arrows in Fig. 1). Five specific residues at the equivalent positions for interacting with and forming the C-terminal aspartyl di-carboxylate clamp with Hsp90 (Russell et al., 1999; Scheufler et al., 2000) are identical or conserved (arginine replacing lysine) in the mammalian UNC-45 TPR domains.

The GC and UNC-45 isoforms are predicted to have molecular weights of 103.450 and 103.41×10^3. Structural prediction programs indicate that the pI of the GC UNC-45 protein would be 6.0, close to that of the *C. elegans* protein. The SM UNC-45 protein is predicted to have a more basic pI of 8.2. The GC and SM UNC-45 proteins are each predicted to consist of about 3:1 α-helical: random coil content with negligible amounts of beta sheet [(Rost, 1996) www.embl-heidelberg.de/predictprotein].

The homologous human and mouse cDNAs for the ubiquitously expressed general cell UNC-45 (see below) that we cloned are represented in the GenBank database as human SMAP1 (direct submission, acc. no. BAB20273.1) and a human colon adenocarcinoma cDNA (direct submission, acc. no. AAH06214**)** and as a mouse cDNA from an induced mammary tumor cDNA (direct submission, acc. no. AAH04717). There is no full-length cDNA in the GenBank database for either the homologous human or mouse striated muscle UNC-45 isoform; (acc. nos. requested). The human SM UNC-45 gene product predicted in the NCBI database (acc. no. XP_091530; gene LOC146862, encoded by acc. no. XM_091530.1) is incorrect. It contains three extra exons encoding 122 additional residues when compared to the SM UNC-45 protein deduced from multiple PCR products.

Radiation hybrid mapping placed the human general cell gene locus on chromosome 15q25-26, correlating well with the human genome project placement of the gene at that location between 96,331,340-96,352,778 bp. The predicted mRNA XM_038413 and protein XP_038413 are in agreement with our cloned cDNA and the appropriate BAC sequences. No human disease loci have yet been mapped near this region.

The mouse GC UNC-45 gene mapped to chromosome 7q14-q21.3 at locus 39 (954788 in Mouse Genome Database, chromosome 7D1 in Map Viewer), as determined by the site of an EST (acc. no. AW538196) that encodes the carboxyl 96

Fig. 1. Alignment of general cell (GC) and striated muscle (SM) isoforms of human (hUNC-45) and mouse (mUNC-45) UNC-45. The TPR domain (red), central region (green) and UCS domain (purple) are indicated. Identity is indicated by black boxes, similarity by grey boxes. The GC and SM isoforms are 55-56% identical, while respective human and mouse isoforms are 94-95% identical. Intron/exon boundaries, indicated by arrowheads (blue for GC, red for SM), are identical for the same mouse and human isoform, and furthermore, are identical for homologous coding regions in GC and SM genes. Red asterisks indicate conserved residues corresponding to *C. elegans* temperature-sensitive UNC-45 mutations (Barral et al., 1998), and blue asterisks indicate *S. pombe* Rng3p mutations (Wong et al., 2000). Blue and red arrows indicate the chymotryptic and tryptic cleavage sites, respectively, present in *C. elegans* UNC-45 (J.M.B. and H.F.E., unpublished).

residues plus 330 bases of 3′ UTR. The loci of the human and mouse *GC unc-45* are syntenic. The mouse coding sequence is within acc. no. XM_124930, on NCBI contig NW_000327.

The human SM UNC-45 gene is entirely contained in a bacterial artificial chromosome (acc. no. AC022916) and mapped to chromosome 17q11, between 33,872 and 33,834 Kb. The SM UNC-45 gene spans 38 kb, and consists of 19 exons. By synteny, the mouse SM UNC-45 gene would be on mouse chromosome 11 at locus 47.5 cM, and is in fact included in a mouse chromosome 11 BAC (acc. no. AL603745). The mouse gene is similar in size to the human gene, 34-kb. No human disease loci have yet been mapped to the SM UNC-45 locus.

Both the human and mouse GC UNC-45 genes are over 10 kb smaller than the SM UNC-45 genes, due to longer introns since total exonic sequences are nearly identical in length. Both GC UNC-45 genes contain one exon more than the SM UNC-45 genes, which encodes the extreme amino terminus. The intron/exon boundaries of the respective mouse and human genes for an isoform are identical, although the introns are of different sizes. The exons of the GC and SM UNC-45 genes are also identical within a species (Fig. 1).

We assembled and aligned predicted UCS domains from the NCBI database (www.ncbi.nlm.nih.gov) (Fig. 2). The 17 full length UCS domains are from the species already mentioned and in addition from *Neurospora crassa*, *Aspergillus fumigatus*, a second genus of nematode, the mosquito

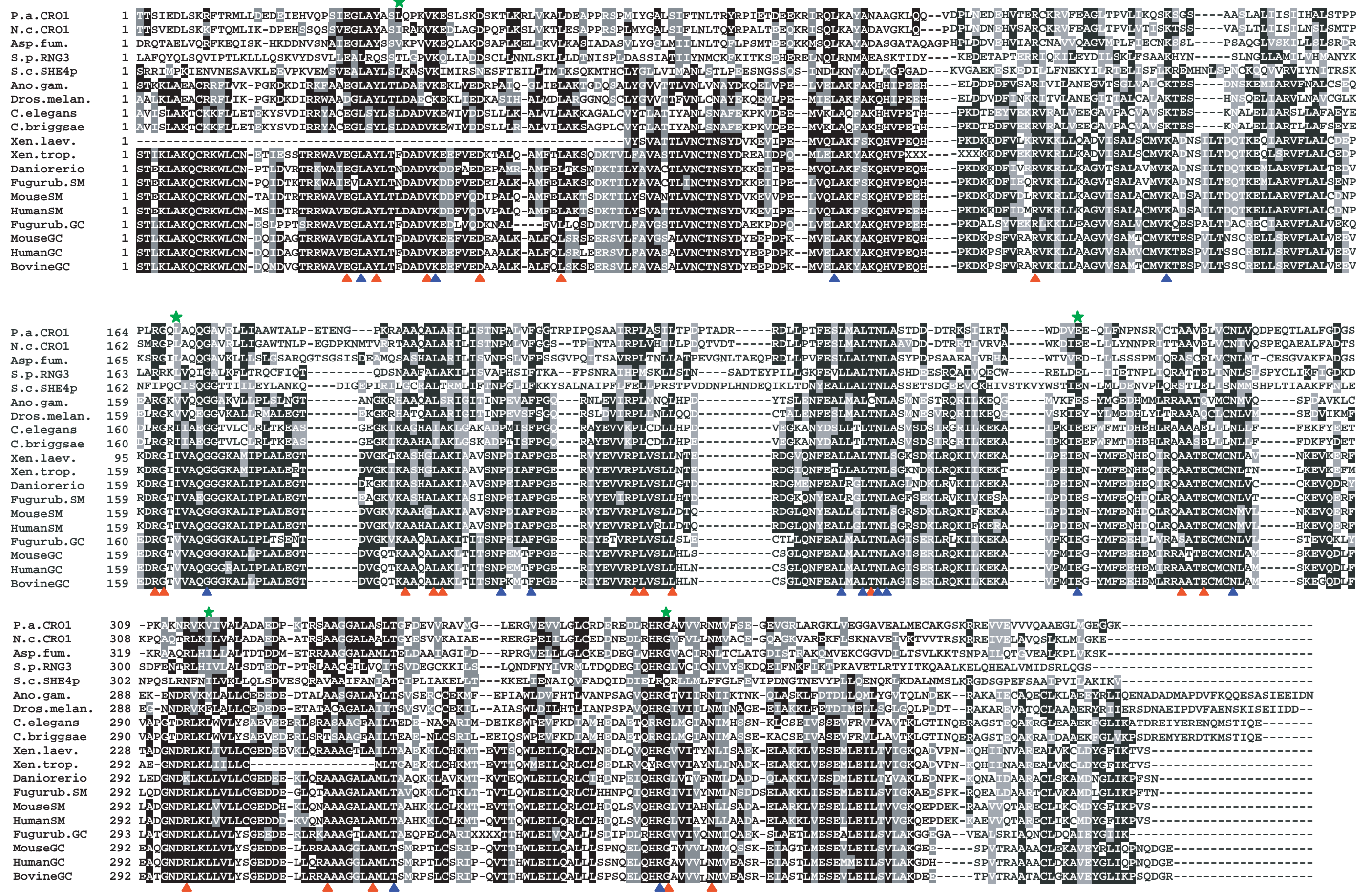

Fig. 2. Alignment of UCS domains from fungal homologues and animal UNC-45 proteins. Only one UCS protein is present in fungi and invertebrates. Two UNC-45 isoforms, SM (striated muscle) and GC (general cell) are present in the bony fish *Fugu rubripies*, suggesting that the gene divergence is ancient. Note that the fungal proteins have extra residues in five places, compared with the animal proteins. Identity is indicated by black boxes, similarity by grey boxes. There is a clear distinction between invertebrate and vertebrate proteins. Blue arrowheads indicate completely conserved residues, and red arrowheads indicate 90-95% identity. Asterisks indicate conserved residues corresponding to *C. elegans* temperature-sensitive UNC-45 (Barral et al., 1998) and *S. pombe* Rng3p mutations (Wong et al., 2000). Ano. gam., *Anopheles gambiae* (coding sequence compiled from acc. no. AAAB01008844.1); Dros. melan., *Drosophila melanogaster* (acc. no. AAK93568); C. elegans, *Caenorhabditis elegans* [(Venolia et al., 1999) acc. no. AAD01976]; C. briggsae, *Caenorhabditis briggsae* [(Venolia et al., 1999) acc. no. AAD01960]; Daniorerio, *Danio rerio* (acc. no. AAL57031); Fugurub., *Fugu rubripies* (SM, coding sequence compiled from JGI Fugu genome project scaffold 6404; GC, coding sequence compiled from JGI Fugu genome project scaffold 465); Human, *Homo sapiens* (SM, coding sequence compiled from AC022916; GC, acc. nos. BAB20273, AAH06214); N.c., *Neuropsora crassa* (direct submission acc. no. T49461*)*; Mouse, *Mus musculus* (SM, coding sequence compiled from acc. no. AL603745; GC, acc. no. AAH04717); P.a., *Podospora anserina* [(Berteaux-Lecellier et al., 1998) acc. no. CAA76144]; S.c., *Saccharomyces cerevisiae* [(Jansen et al., 1996) acc. no. CAA63795]; S.p., *Schizosaccharomyces pombe* [(Wong et al., 2000) acc. no. O74994], Xen. laev., *Xenopus laevis* (coding sequence compiled from acc. nos. AW765658, BJ074261 and BJ091725), Xen. trop., *Xenopus tropicalis* (coding sequence compiled from acc. nos. AL650279, AL656414, AL661438 and AL646713).

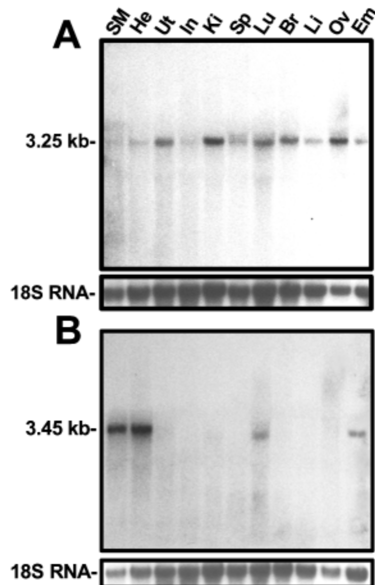

Fig. 3. The two murine UNC-45 mRNAs are differentially expressed. Duplicate northern blots of total RNA from various adult mouse organs and from 12 day embryos were labeled with isoform-specific probes (SM, skeletal muscle; He, heart; Ut, uterus; In, large intestine; Ki, kidney; Sp, spleen; Lu, lung; Br, brain; Li, liver; Ov, ovary; Em, embryo). The GC UNC-45 isoform was detected in all adult organs examined, and the SM UNC-45 isoform was detected in organs consisting mainly of striated muscle. A small amount of GC UNC-45 mRNA was also present in striated muscle tissues. The minor SM UNC-45 band in the lung sample is of unknown origin. Labeling of 18 S RNA indicated comparable loading.

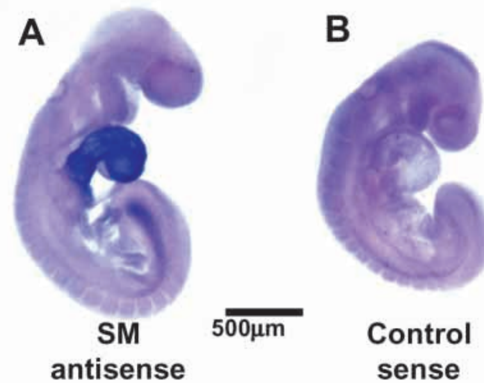

Fig. 4. The SM *unc-45* gene is strongly expressed in the contractile heart during embryogenesis. In situ hybridization was done on whole mouse embryos using gene-specific anti-sense and sense control probes. (A) SM UNC-45 mRNA was detected in the functional heart, as shown here at 8.75 days. (B) Comparison with an 8.5 day embryo labeled with the sense control demonstrates that the SM UNC-45 mRNA was not expressed in other tissues above background level.

Anopheles gambiae, *Drosophila,* the *Danio rerio* zebrafish, the pufferfish *Fugu rubripies*, and cow. Nearly complete UCS domains were assembled for the frogs *Xenopus tropicalis* and *Xenopus laevis*. Blocks of high identity clearly show the divergence of vertebrates from invertebrates. Only 15 residues of the approximately 400 UCS residues are identical in all species studied. The most conserved block includes the sequence LTNL. The fungal proteins have extra residues in five locations, causing gaps in the alignment. The invertebrate UCS domains have C-terminal extensions. The vertebrate UCS domains contain four extra residues corresponding to residues 640-643 and 657-660 in the SM and GC UNC-45 proteins (top right in Fig. 2). These additions and deletions may represent specializations in function.

The presence of two UNC-45 isoforms in the pufferfish gives rise to the notion that the second UNC-45 gene arose sometime during the chordate radiation [see accompanying Commentary (Hutagalung et al., 2002)]. Pair-wise comparisons of identity showed that one of the *Fugu* UNC-45 isoforms is 72% identical and 84% similar to the human and mouse SM UNC-45. The other *Fugu* UNC-45 isoform is 64% identical and 78% similar to the human and mouse GC UNC-45 isoform.

The two murine UNC-45 isoform genes are differentially expressed in adult tissues

The two murine UNC-45 mRNAs are differentially expressed in the adult. GC UNC-45 mRNA was detected in uterus, large intestine, kidney, spleen, lung, brain, liver and ovary using gene-specific labeling of duplicate northern blots containing total RNA from various adult organs (Fig. 3A). The mRNA was relatively less abundant in cardiac and skeletal muscle than in the non-striated muscle tissues. The GC UNC-45 mRNA was also found in whole 12 day mouse embryos. The demonstration of GC UNC-45 mRNA in every tissue examined here confirms the multi-organ expression pattern demonstrated by EST database searches which showed other GC UNC-45 expressing cells or organs include skin, bone marrow, T- cells, urinary bladder, mammary gland, optic nerve, various parts of the eye, germ cells, testis, prostate, pancreas, parathyroid gland and placenta. In addition, over a dozen tumors of various cell-types express GC UNC-45, some to a higher than normal level as suggested by SAGE (serial analysis of gene expression) analysis [see UniGene Cluster Hs.26110 Homo sapiens in the NCBI database (www.ncbi.nlm.nih.gov)].

The SM UNC-45 mRNA is abundant in skeletal muscle and the heart, both of which consist predominantly of striated muscle fibers (Fig. 3B). The same size mRNA was also present in whole embryo samples. However, SM UNC-45 mRNA was not detected in uterus and large intestine, which are rich in smooth muscle cells nor in non-muscle organs such as kidney, liver and ovary. The source of the minor SM reaction in the lung sample is unknown. A TBLASTN query (Altschul et al., 1997) of the EST database reveals that the partial SM UNC-45 sequences are present in cDNA libraries derived mostly from heart and segments of embryo containing the developing heart, such as embryonic body between the diaphragm region and the neck, as well as tissues containing skeletal muscle such as limbs and total head tissue.

The two unc-45 genes are differentially expressed during embryogenesis

To determine the location of early expression of the GC and SM UNC-45 RNA species, in situ hybridization was performed on whole mouse embryos using sense controls and anti-sense RNA probes. The SM UNC-45 gene was strongly expressed in the heart at 8.75 days when it is already beating (Fig. 4A), and was not expressed in other organs above the background level seen in a sense control embryo (Fig. 4B). The GC UNC-45 gene was expressed at high levels in all tissues of an 8-day embryo, the earliest stage embryo obtainable, whereas by 9.75 days the most robust expression was in regions of intense development such as the branchial arches and the forelimb bud (data not shown).

GC UNC-45 and SM UNC-45 mRNAs are differentially expressed during muscle differentiation in vitro

Having determined that the mRNAs for the two isoforms of mammalian UNC-45 are differentially expressed during development and in adult tissues, we addressed when during muscle differentiation the striated muscle isoform is expressed. To assess the relative expression of the two UNC-45 isoform mRNAs, total RNA was isolated from C2C12 cells proliferating in growth medium, and from three stages of muscle differentiation induced by changing confluent cultures to differentiation medium, i.e. fusing myoblasts present at 2 days, young myotubes present at 3.5 days, and older myotubes present after 6 days of differentiation, some of which twitched (Fig. 5A). Total RNA from mouse skeletal muscle and uterus were used as respective positive controls for SM UNC-45 and GC UNC-45 mRNA.

As demonstrated in Fig. 5B, only GC UNC-45 mRNA was expressed in the proliferating C2C12 myoblasts. GC UNC-45 mRNA expression was at the highest level in these cells relative to the differentiating cultures at any stage, as shown by the total pixel number/band normalized to the internal standard of GAPDH (Fig. 5C). SM UNC-45 mRNA was first expressed when aligned myogenic cells were actively fusing (Fig. 5B). This population of fusing myoblasts and the earliest, narrow diameter myotubes expressed GC UNC-45 mRNA at 73% of the level in proliferating cells. Young myotubes in the process of assembling and remodeling myofibrils had the highest relative expression of SM UNC-45, 1.5 times greater than that in fusing myoblasts. The level of GC UNC-45 expression continued to decline during myotube maturation, from 25% in young myotubes to 14% in older myotubes, relative to that in proliferating cells. SM UNC-45 mRNA expression decreased in older myotubes to about half of the maximum found in younger myotubes.

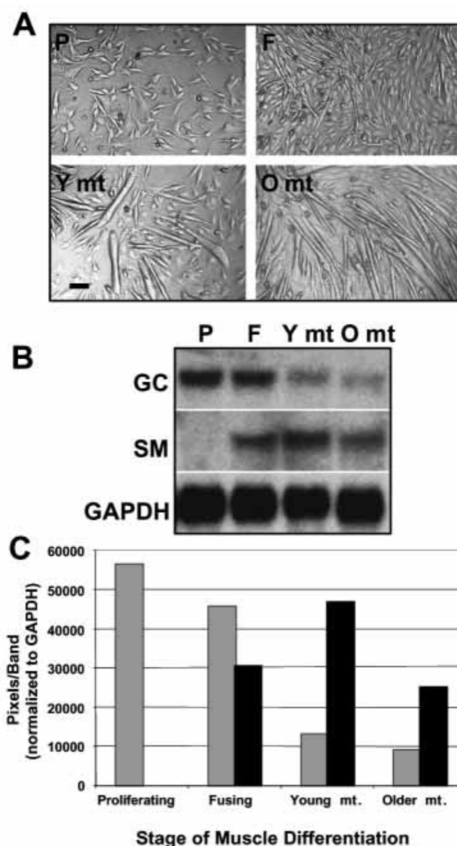

Fig. 5. SM UNC-45 mRNA expression begins when skeletal myogenic cells fuse to form myotubes while expression of GC UNC-45 mRNA decreases in differentiating cultures. (A) Phase-contrast images of C2C12 cultures at different stages of myogenesis; P shows proliferating myoblasts, F shows fusing myocytes, Y mt shows young myotubes, and O mt shows older myotubes. Bar, 20 μm. Total RNA was prepared from these cultures. (B) Differential expression of GC and SM UNC-45 mRNA at the proliferative stage and during the stages of myotube formation, detected in duplicate northern blots. GAPDH mRNA levels show that approximately equal amounts of RNA were loaded per lane. (C) Quantification of the expression levels of GC (gray bars) and SM (black bars) UNC-45 mRNAs at different stages of in vitro muscle differentiation, shown as pixels per band normalized to the levels of GAPDH mRNA per sample (mt, myotube cultures).

GC UNC-45 functions in cell proliferation

Antisense experiments were performed to test whether the GC and SM UNC-45 isoforms exhibited different functions. The differential tissue expression of the two isoforms suggested that the GC UNC-45 may have a role in cytoskeletal functions and the SM UNC-45 may have a more specialized role in sarcomere assembly and function. Therefore the antisense treatments of C2C12 cells focused on cell proliferation and muscle differentiation. For studies of the effects of suppression of UNC-45 isoform mRNA in proliferation, C2C12 cells were plated at low density and antisense oligonucleotides were added daily to 2.5 μM for three days. This concentration permitted only partial mRNA suppression. However, higher concentrations of oligonucleotides had obvious toxic effects, evidenced by cell death in control cultures.

The extent of suppression of UNC-45 mRNA expression by treatment with antisense oligonucleotides was determined by northern blotting. Three days of treatment of proliferating C2C12 cultures with 2.5 μm GC UNC-45 antisense oligonucleotides reduced GC UNC-45 mRNA expression to half the amount in the control or SM UNC-45 antisense treated cultures (Fig. 6A). Cell proliferation was judged from total DNA as determined by fluorescence (Jones et al., 2001). The GC UNC-45 antisense oligonucleotides suppressed cell proliferation to 68-75% of values obtained by treatment with the control reverse GC oligonucleotide within 3 days of treatment (Fig. 6B,C). This reduction is significant (P=0.00008). Treatment with the SM UNC-45 antisense oligonucleotides did not significantly effect the proliferation rate (P=0.36). Cell viability was not affected by 2.5 μM oligonucleotide, as assessed by all cultures having less than 1% of trypan blue-stained cells.

SM UNC-45 and GC UNC-45 have distinct roles in muscle cell differentiation

To gain insight into the role of each UNC-45 isoform in muscle differentiation, C2C12 cells were treated with 2.5 μM antisense oligonucleotides throughout the proliferative phase and for up to a week of differentiation. At this point, twitching myotubes with robust striations were present in cultures receiving none or negative control oligonucleotides. The SM UNC-45 antisense treatment specifically reduced SM UNC-45 mRNA expression

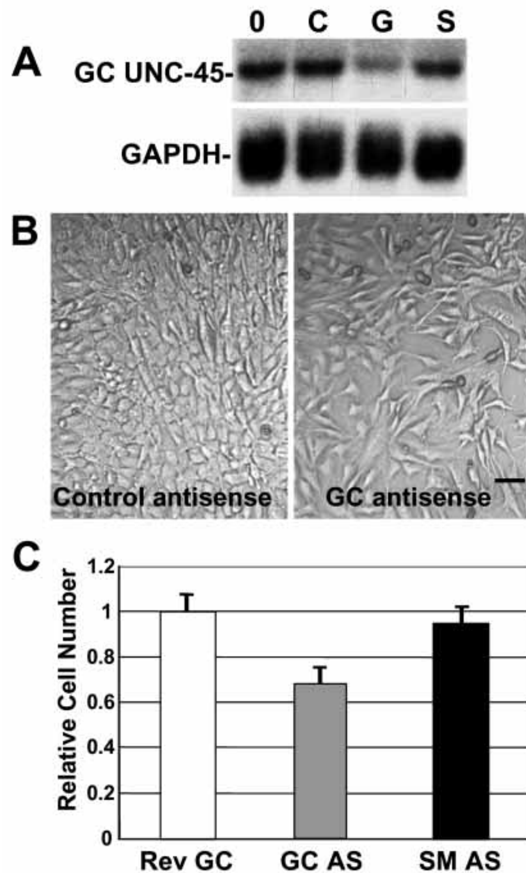

Fig. 6. Cell proliferation was retarded when levels of GC UNC-45 mRNA were reduced. (A) Treatment of C2C12 cultures with 2.5 μM GC UNC-45 antisense oligonucleotides suppressed GC UNC-45 mRNA expression by 50%. Northern blot of GC UNC-45 mRNA in 10 μg of total RNA from proliferating cells treated 3 days with none (0), a negative control (C), GC UNC-45 antisense (G) or SM UNC-45 (S) antisense oligonucleotide. Note that SM UNC-45 mRNA is not expressed in proliferating cells (see Fig. 5). GAPDH labeling demonstrated equal RNA loads per lane. (B) Phase-contrast images of proliferating C2C12 cells treated with a negative control or GC UNC-45 antisense oligonucleotide, demonstrating reduced proliferation in GC UNC-45 antisense treated cultures. Cells treated with no or SM UNC-45 antisense oligonucleotide resembled control cultures. Bar, 50 μm. (C) The relative reduction in cell proliferation caused by 3 days of GC or SM UNC-45 antisense treatment, determined from pair-wise comparisons with cultures treated with the control oligonucleotide. Total DNA content was used to gauge cell number. The mean of five experiments is given ±s.d.

to half the control levels (Fig. 7A). SM UNC-45 mRNA is expressed at control levels in the GC antisense-treated cultures, reflecting their limited differentiation (Fig. 7A).

For quantitative analysis of differences in myotube and sarcomere formation in C2C12 cultures treated with antisense oligonucleotides, over 600 cells from each treatment were scored for the number of nuclei per cell, expression of muscle-specific sarcomeric α-actinin as viewed in low magnification, and the pattern of staining of sarcomeric α-actinin viewed in high magnification. GC UNC-45 antisense treatment severely reduced myoblast fusion so that the positively stained cells contained predominantly one to four nuclei (Fig. 7B,D). Treatment with the SM UNC-45 antisense oligonucleotide reduced fusion but to a lesser extent. These cells were able to fuse to form multinuclear myotubes containing over a dozen nuclei (Fig. 7B,D). Reduction of SM UNC-45 mRNA affected sarcomere formation more directly, so that about half the myotubes had only small submembraneous structures with striated α-actinin while the interior of the myotubes was largely unstriated (Fig. 7C).

Considering the paucity and instability of thick filaments in *C. elegans unc-45* temperature-sensitive mutants (Barral et al., 1998), we addressed whether reduction of SM UNC-45 had an effect on the total amount of skeletal myosin heavy chain in the C2C12 myotube cultures. Skeletal muscle myosin heavy chain (MHC) was specifically detected by immunoblotting using MF 20 antibody against equivalent amounts of total protein from myotube cultures treated 8 days plus or minus negative control or antisense oligonucleotides directed against GC or SM UNC-45. Reduction of SM UNC-45 mRNA had no significant effect on the amount of skeletal MHC in the myotube cultures compared to untreated and negative control antisense-treated cultures (Fig. 8). GC UNC-45 antisense-treated cultures had less than half as much skeletal MHC as the other samples, consistent with the suppression of myotube formation (Fig. 8). In these cultures skeletal MHC was derived from the small myotubes containing two to four nuclei and the rare larger myotubes, which were positively stained by the MF20 antibody, in contrast to the larger multi-nucleated myotubes in controls (see Fig. 7B). The effects of antisense suppression upon sarcomere formation therefore do not appear to be related to changes in the amounts of MHC or other major proteins.

Discussion

UNC-45 proteins throughout the animal kingdom have the same three-region structure of the canonical *C. elegans* protein, with an amino-terminal TPR domain consisting of three tandem TPR motifs, a central region that so far is unique to UNC-45 proteins and a C-terminal UCS domain. In contrast to flies and worms, there are two isoforms of UNC-45 in vertebrates, ranging from the pufferfish to humans. The mammalian UNC-45 isoforms have the same predicted molecular weight, but differ in predicted pI. The encoding genes show distinct chromosomal location and are differentially expressed in both embryonic and adult tissues. The general cell isoform appears to be ubiquitously expressed, while the striated muscle isoform was found only in cardiac and skeletal muscle.

The C2C12 skeletal myogenic cell line allows one to examine general cytoskeletal functions in the proliferative stage as well as muscle-specific functions during differentiation. Consistent with its widespread expression in organs, GC UNC-45 mRNA is expressed in proliferating, non-differentiated myoblasts. This expression level is greater than any stage of muscle differentiation. By the time more robust myotubes have developed, the levels of GC UNC-45 mRNA are only 14% of those in proliferating cells. Reducing the GC UNC-45 mRNA to about a half-normal level by antisense treatment decreased C2C12 cell proliferation to 68-75% of control values. The GC UNC-45 antisense treatment also inhibited myoblast fusion. In contrast to the GC isoform, SM UNC-45 mRNA was not expressed until myogenic cells started fusing, with the highest levels of expression in young myotube cultures. Assembly and remodeling of myofibrils is highest

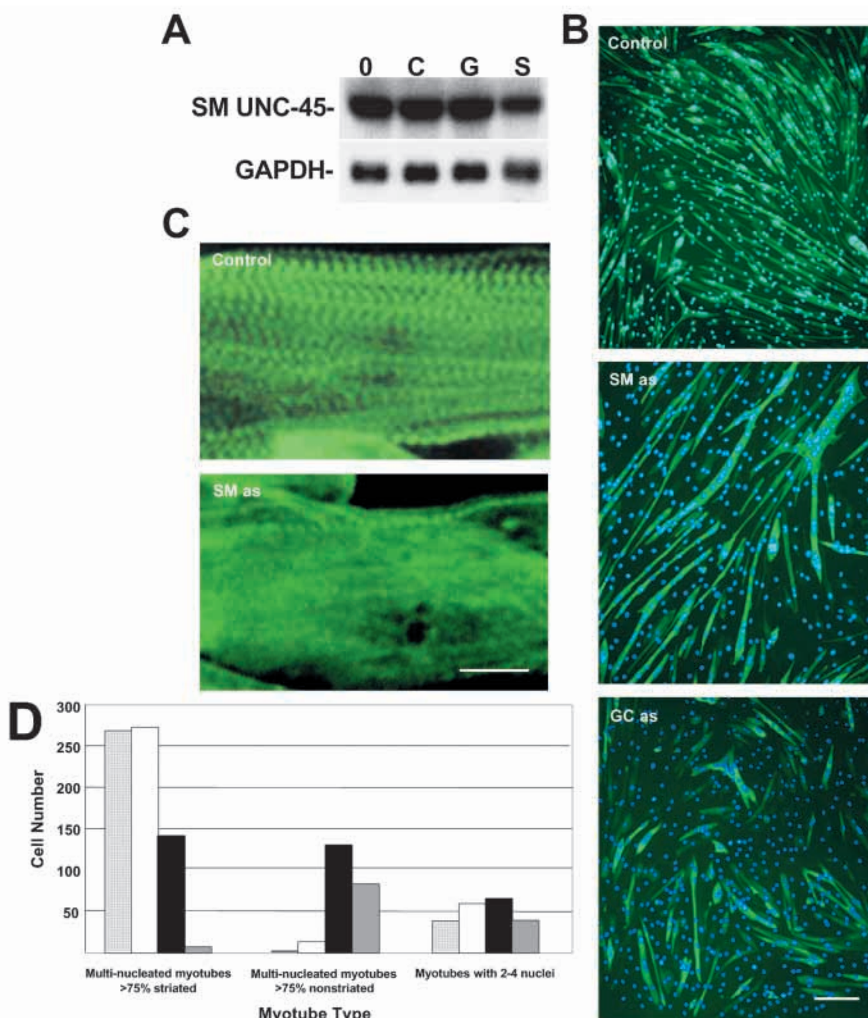

Fig. 7. GC and SM UNC-45 have different functions in muscle differentiation. C2C12 skeletal myocytes were treated for 8 days with none (0), negative control (C), GC UNC-45 antisense (G) or SM UNC-45 (S) antisense oligonucleotides. (A) SM UNC-45 antisense treatment specifically reduced SM UNC-45 mRNA expression by 50%. Northern blot of 10 μg total RNA from myotube-containing cultures treated for 8 days, labeled with a SM UNC-45 and a GAPDH probe. SM UNC-45 mRNA was expressed at control levels in the GC UNC-45 antisense-treated cultures, reflecting the presence of short myotubes. (B) Muscle differentiation was assessed by immunostaining with EA53 antibody to sarcomeric α-actinin. Nuclei were stained blue with DAPI. Unstained cells appear black with blue nuclei. Robust, multinucleated myotubes were observed in cultures treated with the negative control oligonucleotide. Cultures not treated were indistinguishable from these. SM UNC-45 antisense-treated cultures had fewer myotubes than controls. The majority of differentiated cells in GC UNC-45 antisense-treated cultures were short myotubes with one to four nuclei, suggesting that GC UNC-45 is necessary for normal levels of myoblast fusion. Bar, 100 μm. (C) Subnormal levels of SM UNC-45 reduced the extent of striated myofibrils, as shown by this high magnification view of sarcomeric α-actinin staining in myotubes from negative control and SM UNC-45 antisense-treated cultures. Bar, 10 μm. (D) Graphical representation of the effects of GC and SM UNC-45 antisense treatment on myotube differentiation in vitro. Bar colors are: stippled for no treatment; white for control oligonucleotide; black for SM antisense; and grey for GC antisense. Six hundred cells were counted for each sample; those not represented here were mononuclear. GC UNC-45 appears to function in cell fusion while SM UNC-45 has a role in fusion and formation of striated myofibrils within myotubes.

during young myotube formation, consistent with SM UNC-45 being involved in the process of thick filament assembly and sarcomere formation. Unlike the GC UNC-45 antisense-treated cells, cells treated with antisense SM UNC-45 oligonucleotides were able to fuse and form myotubes. However, half of these myotubes lacked striated myofibrils, as identified by staining for sarcomeric α-actinin.

The SM to GC UNC-45 mRNA expression ratio in older myotube cultures was 2.8. This ratio contrasts with the 18 to 1 ratio of SM to GC UNC-45 mRNA in adult mouse skeletal muscle. This difference is most likely a consequence of only partial differentiation in cell cultures versus in vivo. The basis for less SM UNC-45 mRNA in older as compared to younger

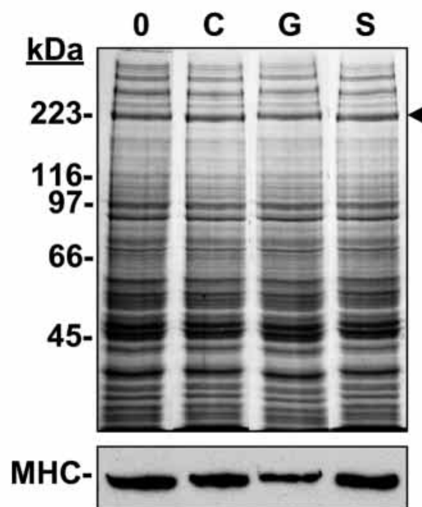

Fig. 8. Reduction of SM UNC-45 mRNA did not alter skeletal myosin expression in myotube cultures. A Commassie blue-stained 7.5% polyacrylamide gel shows equal amounts of total protein from myotube cultures treated for 8 days with none (0), a negative control (C), GC UNC-45 antisense (G) or SM UNC-45 (S) antisense oligonucleotide. Myosin heavy chain (MHC) (arrow) and molecular weight markers are indicated. The lower panel shows the results of immunoblotting with MF 20 antibody to specifically detect skeletal MHC in the samples above. Equal amounts of skeletal MHC were in all but the GC UNC-45 antisense-treated cultures, which contained more myoblasts and few large myotubes.

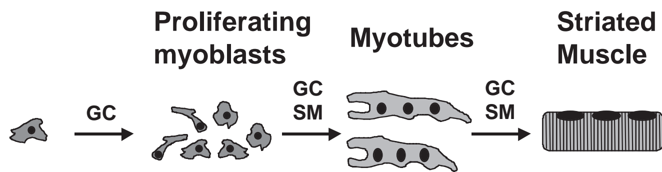

Fig. 9. Schematic summary of the proposed roles of GC and SM UNC-45, based on the processes inhibited by antisense reduction of specific mRNAs. GC UNC-45 functions in cytoskeletal processes in proliferating, non-differentiating cells. Both GC and SM UNC-45 function in myotube formation through cell fusion. Myofibril formation requires both GC and SM UNC-45, consistent with the fact that the cytoskeleton is necessary for the development and maintenance of organized myofibrils.

myotube cultures is unknown. However, protein half-life may vary and in addition the relationship between protein and mRNA expression need not be proportionate. This relationship can be determined for the UNC-45 isoforms when specific antibodies become available. Further work is needed to determine whether both GC and SM UNC-45 are present in striated muscle fibers. The antisense results correlate with the mRNA expression patterns indicating that GC UNC-45 has a role in processes involving the cytoskeleton and SM UNC-45 has a more muscle-specific function in sarcomere assembly. We cannot rule out the possibility of functional overlap between the two isoforms because suppression of either isoform had an affect on cell fusion and sarcomere formation. To date, it is not known what myosins are involved in the process of myocyte fusion. Based on their mRNA expression and the different roles of the two UNC-45 isoforms in C2C12 myogenesis, these two isoforms most likely have separate and distinct activities, possibly mediating functions that involve different classes of myosin. However, either isoform may function in myosin folding, assembly, and/or contractile activity.

Previous studies have shown that proteins in the UCS domain family are required for a variety of myosin- and actin-based processes utilizing both conventional and unconventional myosins. She4p is required for mRNA transport involving an unconventional myosin type V in *S. cerevisiae* (Beach and Bloom, 2001; Jansen et al., 1996). Temperature-sensitive mutations in the essential gene *rng3*, a member of the UCS family, block the assembly of the actomyosin ring during cytokinesis, and are synthetically lethal with mutations in the cytoskeletal myosin II gene (Balasubramanian et al., 1998). *C. elegans* UNC-45 has been shown to directly bind the head of muscle myosin II (Barral et al., 2002), and to interact with cytoskeletal type II and unconventional type V myosins through two-hybrid analysis (W. Ao and D. Pilgrim, personal communication). In addition, *C. elegans* UNC-45 was recently shown to be a myosin-targeted chaperone since it prevents the thermally induced aggregation of the myosin head (Barral et al., 2002). This finding, in conjunction with UNC-45 binding the chaperone Hsp90, suggests that UNC-45 might influence thick filament assembly through a role in myosin folding. The other UCS domain proteins may function likewise for different myosin substrates. In this regard, it is significant that wild-type *S. pombe* Rng3p is sequestered only by *myo2-E1* myosin II, which has a mutation in the myosin head and leads to defective contractile ring assembly (Wong et al., 2000).

Based on their mRNA expression and the different effects of suppression of the two UNC-45 isoforms in C2C12 muscle differentiation, we propose that they are involved in distinct functions. The GC UNC-45 isoform appears to have more of a general function, possibly being involved in cell division, whereas the SM isoform is related to striated muscle differentiation including myofibril formation. The functions of the two isoforms are not necessarily independent of one another, because the cytoskeleton is needed for formation and maintenance of myofibrils. Lack of cytoskeletal proteins such as vinculin (Barstead and Waterston, 1991), talin (Moulder et al., 1996), desmin (Li et al., 1997; Milner et al., 1996), certain isoforms of integrin (Gettner et al., 1995; Moorthy et al., 2000; Volk et al., 1990) and spectrin (Hammarlunda et al., 2000; Norman and Moerman, 2002) as well as the extracellular matrix protein perlecan (Rogalski et al., 1995) leads to defects in myofibril formation and maintenance. Therefore both UNC-45 isoforms may be necessary for functional sarcomeres.

Our results are consistent with a model (Fig. 9) in which the two UNC-45 isoforms have separate, but possibly overlapping functions in striated muscle differentiation. In this model, GC UNC-45 would be involved in cell proliferation and cytoskeletal maintenance of myofibrils once they have formed. SM UNC-45 would function in the development of sarcomeres. These two proteins may interact with different myosins in their respective functions.

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