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 Huta galung 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 tetra repeat (TPR), a ~400 residue central region and a ~400 residue UNC-45/Cro1/She1/4p (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-
Materials and Methods

Identification of mammalian UNC-45 cDNAs and cloning

Expressed sequence tagged (EST) fragments of mouse or human UNC-45 cDNA 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 gene. A putative 5’-UTR, and was the product of primers 5’-CTC ACC GTG CCA CAC C3’ and 5’-GAG TCT GCC ACG 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 AGC CAA CCG AAT GCA TGT G3’ and 5’-CAG TCT ACA GCC GTG TAT CGC TGG TGC-3’. Twenty-five ng of each probe was labeled with [32P]-dCTP (Amersham, Piscataway, N J) by a random nonomer method to specific activities of 5 · 10¹⁰–1 · 10¹¹ cpm/mg, and used at 1 · 10¹⁰ 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); 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 (18 S RNA and GAPDH) at –80°C. Films were digitized by scanning, and Photoshop (Adobe 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 UNSCAN-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 digoxigenin-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; Silverstein 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% CO₂ 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 1:40 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 CCTG-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 trypanized cells resuspended in trypsin 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 2x 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 16x10⁵ 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 Na₂HPO₄, 1.8 mM KH₂PO₄, 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 5x 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 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 almost identical at the amino acid sequence level.
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 numbering referring to that protein, unc-45 b131 (G427E), su2002 (L559S), m94r/v450 (E781K) and c268 (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 phenylalanine 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 pl 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 pl 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](http://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_0091530; 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
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 isofrom 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...
domains were assembled for the frogs. Demonstration of GC UNC-45 mRNA in every tissue examined was also found in whole 12 day mouse embryos. The GC UNC-45 mRNA was relatively less abundant in cardiac and skeletal muscle than in the non-striated muscle tissues. The 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 not detected in other tissues above background level. The minor SM UNC-45 band in the lung sample is of unknown origin. Labeling of 18 S RNA indicated comparable loading.

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 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.
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.

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.

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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 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 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 MF20 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.

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Mammalian cytoskeletal and muscle UNC-45 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. 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.

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.
myosin substrates. In this regard, it is significant that wild-type UCS domain proteins may function likewise for different filament assembly through a role in myosin folding. The other 2002). This finding, in conjunction with UNC-45 binding the thermally induced aggregation of the myosin head (Barral et al., 2002), shown to be a myosin-targeted chaperone since it prevents the possible mediation of chaperone function during 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 type II and unconventional type V myosins through two-hybrid analysis (W. Ao and D. Pilgrim, personal communication). In addition, 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 has been 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 (Gumbiner et al., 1995; Moorthy et al., 2000; Volk et al., 1990) and spectrin (Hammarlund et al., 2000; Norman and Moerman, 2002) as well as the extracellular matrix protein perlecan (Poy 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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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 cytokinesis is necessary for the development and maintenance of organized myofibrils.

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