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Archvillin, a muscle-specific isoform of supervillin, is an early expressed component of the costameric membrane skeleton

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Summary

The membrane skeleton protein supervillin binds tightly to both F-actin and membranes and can potentiate androgen receptor activity in non-muscle cells. We report that muscle, which constitutes the principal tissue source for supervillin sequences, contains a ~250 kDa isoform of supervillin that localizes within nuclei and with dystrophin at costameres, regions of F-actin membrane attachment in skeletal muscle. The gene encoding this protein, 'archvillin' (Latin, archi; Greek, árchos; 'principal' or 'chief'), contains an evolutionarily conserved, muscle-specific 5′ leader sequence. Archvillin cDNAs also contain four exons that encode ~47 kDa of additional muscle-specific protein sequence in the form of two inserts within the function-rich N-terminus of supervillin. The first of these muscle-specific inserts contains two conserved nuclear targeting signals in addition to those found in sequences shared with supervillin. Archvillin, like supervillin, binds directly to radiolabeled F-actin and co-fractionates with plasma membranes. Colocalization of archvillin with membrane-associated actin filaments, non-muscle myosin II, and – to a lesser extent – vinculin was observed in myoblasts. Striking localizations of archvillin protein and mRNA were observed at the tips of differentiating myotubes. Transfected protein chimeras containing archvillin insert sequences inhibited myotube formation, consistent with a dominant-negative effect during early myogenesis. These data suggest that archvillin is among the first costameric proteins to assemble during myogenesis and that it contributes to myogenic membrane structure and differentiation.

Key words: Costamere, Sarcolemma, Membrane skeleton, C2C12 cells, 50MB-1 cells

Introduction

Organized complexes of membrane-associated cytoskeletal proteins (membrane skeletons) maintain the integrity and organization of the plasma membrane (sarcolemma) of striated muscle cells during the mechanical stresses associated with load-induced stretching and muscle contractions (reviewed by Benjamin and Ralphs, 2000; Berthier and Blaineau, 1997; Small et al., 1992; Stromer, 1995; Watkins et al., 2000). For example, longitudinal mechanical stresses are transduced through membrane skeletons associated with myotendinous junctions – highly invaginated areas of contact between tendons and the ends of muscle cells. Lateral forces across the sarcolemma to the basal lamina are resisted primarily by costameres, cables of filamentous cytoskeletal material that connect the sarcolemma to Z- and M-lines in underlying myofibrils (Danowski et al., 1992; Pierobon-Bormioli, 1981; Street, 1983). Costameres were originally defined immunocytologically as a rib-like lattice containing vinculin, spectrin and γ-actin (Craig and Pardo, 1983; Pardo et al., 1983). These structures also contain dystrophin (Gossrau, 1998; Porter et al., 1992; Rybakova et al., 2000; Zhou et al., 1998),

dystrophin-associated glycoproteins (Rahkila et al., 2001; Rybakova et al., 2000), integrins (Carver et al., 1994; McDonald et al., 1995; Terracio et al., 1989), α-actinin (Danowski et al., 1992), talin (Belkin et al., 1986), ankyrin (Nelson and Lazarides, 1983) and desmin (Tidball, 1992).

The costamere-associated sarcolemmal membrane has been proposed to consist of a mosaic of domains (Rahkila et al., 2001; Williams and Bloch, 1999a) containing dystrophin, integrins, spectrin and associated proteins, and cholesterol-rich, low-density membrane domains called caveolae. Caveolae are 50-100 nm flask-shaped membrane invaginations implicated in signal transduction and endocytosis (Anderson and Jacobson, 2002). Muscle caveolae are enriched in caveolin-3 (Tang et al., 1996; Way and Parton, 1996), a muscle-specific protein that cofractionates and coprecipitates with dystrophin and associated proteins (Song et al., 1996; Sotgia et al., 2000). Although many component proteins have been identified and interactions between costameric proteins have been described, additional interactions between the lateral sarcolemma and the cytoskeleton are suggested by the partial retention of costamere structure and/or function after loss of,

for instance, dystrophin or caveolin-3 (Galbiati et al., 2001; Williams and Bloch, 1999b). Thus, the complete composition and regulation of costameres during muscle functioning have yet to be elucidated.

As part of our ongoing analyses of actin-based membrane skeletons in plasma membranes of motile cells (Luna et al., 1997), we have identified and isolated a neutrophil membrane complex containing non-erythrocyte spectrin (fodrin), actin, non-muscle myosin-IIA and a novel, ~205 kDa membrane protein (Nebl et al., 2002; Pestonjamasp et al., 1995; Pestonjamasp et al., 1997). This complex is enriched in cholesterol and contains associated signaling proteins and integral proteins characteristic of low-density membrane domains (Nebl et al., 2002). The 205 kDa protein, named supervillin because of C-terminal sequence similarities to the microvillar protein villin (Bretscher and Weber, 1979), binds directly to F-actin, promotes actin filament bundling in vivo and resists extraction from neutrophil membranes with high pH carbonate buffers (Nebl et al., 2002; Pestonjamasp et al., 1995; Pestonjamasp et al., 1997). Thus, supervillin is implicated as an actin-membrane linkage protein in a membrane skeleton associated with cholesterol-rich, low-density membrane domains.

Supervillin has been implicated in the direct or indirect control of cell adhesion. In confluent, non-proliferating Madin-Darby bovine kidney (MDBK) cells, supervillin localizes with E-cadherin at sites of cell-cell adhesion and is internalized with adherens junctions proteins in ring-like structures during EGTA-induced release of intercellular contacts (Pestonjamasp et al., 1997). Overexpression of full-length supervillin or N-terminal supervillin sequences in COS7 and CV1 cells disrupts the integrity of focal adhesion plaques (Wulfkuhle et al., 1999), and comparable levels of supervillin overexpression are found in several carcinoma cell lines (Pope et al., 1998). Also, supervillin has been isolated as part of a protein complex with laminin α3, integrin β2, the P2X$_7$ ATP-gated ion channel, and receptor protein tyrosine phosphatase-β (Kim et al., 2001), further suggesting cross-communication with proteins involved in cell-substrate attachment and/or motility.

Supervillin may also contribute to nuclear architecture and function. Functional nuclear targeting sequences in the N-terminal and central domains of supervillin target chimeric proteins into detergent-resistant structures within the nuclei of COS7 cells (Wulfkuhle et al., 1999). Cell-cycle-based control of one or more of these nuclear targeting sequences is probably responsible for the localization of endogenous supervillin to MDBK cell nuclei during active cell proliferation (Pestonjamasp et al., 1997). Supervillin is also a transcriptional activator of the androgen receptor (Ting et al., 2002) and has been implicated in the testosterone-mediated cessation of dermal papilla cell proliferation (Pan et al., 1999), suggesting the possibility of other roles during cell growth.

Quantification of supervillin message levels in human tissues indicate that cross-hybridizing mRNAs are most abundant in human tissues rich in striated or smooth muscle (Pope et al., 1998). The highest message levels are found in skeletal muscle, bladder, heart and aorta. In the current study, we have investigated the nature of the supervillin-related protein in muscle by cloning and characterizing a ~250 kDa muscle isoform of supervillin from human and mouse skeletal muscle. This protein is derived from the supervillin genomic locus (SVIL) by differential splicing of five conserved exons, four of which encode muscle-specific protein sequences distributed as two 'inserts' within the function-rich N-terminus of the protein. Because of the likelihood of muscle-specific conserved functions, we suggest that the isoform of supervillin found in muscle, the principal source of supervillin in the body, be called 'archvillin' (Latin, archi; Greek, árchos; 'principal' or 'chief').

Materials and Methods

Antibody preparation
Affinity-purified anti-H340 antibodies (α-H340)
Polyclonal antisera were generated against the first 340 residues of human supervillin. The HSV41 clone (Pope et al., 1998) containing human supervillin cDNA sequences (accession no. #AF051851) was digested with *Eco*RI and *Not*I and ligated into the pGEMEX-1 vector (Promega Corp., Madison, WI). A chimeric fusion protein consisting of 260 amino acids of T7 gene10 and linker sequence plus amino acids 1-340 of human supervillin (H340) was expressed after isopropyl-β-D-thiogalactopyranoside (IPTG) induction in BL21(DE3) bacteria, purified as inclusion bodies, and used as an immunogen for the production of rabbit polyclonal antisera (Research Genetics, Huntsville, AL). Antibodies specific for H340 were affinity-purified against a similarly generated fusion of H340 with glutathione *S*-transferase (GST) in the pGEX-6p-1 vector (Amersham Pharmacia Biotech, Piscataway, NJ).

Anti-pepA antibodies (α-pepA)
Polyclonal antisera directed against amino acids 900-918 of bovine supervillin were prepared as described (Pestonjamasp et al., 1997).

Cell culture and transfection
Helen Blau (Stanford University, Palo Alto, CA) kindly provided 50MB-1 human myoblasts (Webster et al., 1988). Cells were grown at subconfluent densities in Ham's F-10 media supplemented with 20% fetal calf serum (FCS) and 1% v/v chick embryo extract (60 Å ultrafiltrate; Gibco-BRL, Gaithersburg, MD), with medium changes every other day. To induce differentiation, cultures were grown to near confluence and then maintained in Dulbecco's modified Eagle's medium (DMEM)-low glucose (Gibco-BRL), 5% horse serum, 0.3 μM insulin and 1 μM dexamethasone (Sigma) without further medium changes until the appearance of myotubes. Human diploid fibroblasts WI-38 and cervical carcinoma HeLa cells (ATCC) were grown in DMEM-high glucose (DMEM/HG; Gibco-BRL) supplemented with 10% FCS and gentamycin.

The C2C12 mouse skeletal muscle cell line was a gift from Janet and Gary Stein (University of Massachusetts Medical School, Worcester, MA). Cells were maintained in DMEM/HG supplemented with 10% FCS and penicillin/streptomycin. Differentiation was induced in 2% horse serum, DMEM/HG for 4 to 6 days (Huang et al., 2000). Myoblasts (50MB-1 and C2C12 cells) were transfected using the Effectene Transfection kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The effects of murine archvillin deletion proteins on differentiation of C2C12 were measured as the percentage of the total number of transfected cells present as myotubes after 6 days of incubation in differentiation medium.

Muscle preparations and immunoprecipitations
Rabbit muscle fractions
Crude rabbit skeletal muscle plasma membranes were prepared from freshly dissected or frozen back and leg muscles by flotation through 30% (w/v) sucrose, as described (Ohlendieck et al., 1991). The

Table 1

Archvillin PCR primers

Name	Human	Name	Murine
H96-2R	5′-GGAGGTTTCTGTGCAGTATTTGGATCGAGTT-3′	MSV-F1	5′-YTGCACTYCCTAAAGYTGCAGAATTAAGAMAAAT-3′
HU5P-GAP	5′-GACCTGTCCTGCAGACGGCGTAGCCTC-3′	MSV-R1	5′-ATGGTTTTACAGAGCTTGGCTAATACATCTTCCA-3′
HSK-P1F	5′-CTTTTGGGATGTAGCCTTTACCTGTTTCCTGAA-3′	MAV-R296	5′-TTTGTATCTTGCGATCCTTTCCGCTTTGGACT-3′
HSK-P1R	5′-GTGGAAAGAAGTTTCCAAACAGTTCCCTTGAAC-3′	MAV-R121	5′-AGGGTCTGTGGCACGCATGTATCT-3′
HSK-P2F	5′-GTACAGGGAAACCCAAACATGAGTGGTTTCTCC-3′	CRATY-F	5′-ATGACAAACTCGTTCCTGATGATGACTACTGGG-3′
HSK-P2R	5′-TGCCTAGCTGGAACAAGAAGGCAGTGCTATCTA-3′		

PCR primers for EGFP-tagged murine archvillin constructs

Constructs	Forward primers	Reverse primers
EGFP-Insert 1	5′-<u>CTCGAG</u>AGTACAGGGAAGCCCACACATGAGTGGT-3′	5′-<u>CCGCGG</u>CCTTATCCGACTCCTTTCGCTCTGCT-3′
EGFP-MAV	5′-<u>CTCGAG</u>ATGAAAAGAAAAGAAAGAATTGCTAGGCG-3′	5′-<u>CCGCGG</u>TCAGAATAGGCCCTTTGACTTCTTCAGG-3′
	*Xho*I	*Sac*II

following protease inhibitors (Sigma Chemical Co., St Louis, MO) were included in all buffers: 1 µg/ml aprotinin, 1 µM pepstatin A, 0.5 µg/ml leupeptin, 1 mM benzamidine, 1 µM antipain, 1 mM PMSF. Equivalent amounts of protein in the low-density fraction, which was enriched in plasma membranes, and in the higher-density fraction, which was enriched in sarcoplasmic reticulum and T-tubules, were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

Muscle extracts

Murine hind leg muscles, or rabbit back and leg muscles, were ground under liquid N_2 and extracted twice with 1% SDS for 10 minutes at 70°C. Extracts either were denatured with sample buffer for SDS-PAGE or were diluted 10-fold with 1% Triton X-100 in PBS for immunoprecipitations. Triton X-100 extracts from ~0.3 mg muscle were pre-cleared for 2 hours at 4°C with rabbit immunoglobulin (RIgG) bound to protein A/G beads (Pierce Chemical Company, Rockford, IL) and incubated with 20 µg RIgG or α-H340 bound to protein A/G beads for 5 hours at 4°C. Beads were washed extensively with PBS. Bound immunoprecipitated proteins were eluted by heating for 5 minutes at 95°C in SDS sample buffer and analyzed by α-H340 immunoblot and F-actin blot overlay.

Immunoblots

Proteins were separated by SDS-PAGE (Laemmli, 1970) and electrotransferred to nitrocellulose (0.45 µm pore size) (Schleicher and Schuell, Keene, NH). Nitrocellulose blots were blocked with 5% nonfat powdered milk and probed with primary antibodies for 2 hours at room temperature or overnight at 4°C. Concentrations of primary antibodies were as follows: 10 µg/ml affinity-purified α-H340, 20 µg/ml α-pepA, 5 µg/ml anti-caveolin 3 (BD Transduction Laboratories, San Diego, CA), 1:20 dilution of anti-dystrophin (Novocastra Lab, Burlingame, CA). Interacting polypeptides were visualized using either ^{125}I-labeled protein A or protein A conjugated to horseradish peroxidase and an ECL substrate kit (KPL, Gaithersburg, MD). Reactive polypeptides were detected by exposure to Biomax-MS X-ray film (Eastman Kodak, Rochester, NY). For double labeling with radioactively labeled F-actin, anti-rabbit antibody conjugated to alkaline phosphatase was used with a BCIP/NBT substrate kit (KPL, Gaithersburg, MD) for colorimetric detection.

F-actin blot overlay

For F-actin overlays, ^{125}I-labeled actin was polymerized in the presence of rabbit gelsolin, stabilized with phalloidin and used at a final concentration of 50 µg/ml in 5% nonfat powdered milk (Luna, 1998). In some experiments, actin was labeled with [α-^{32}P]ATP (Mackay et al., 1997), using 1 mg of actin and 1 mCi of [α-^{32}P]ATP. Nitrocellulose blots were exposed to film for 5 days at –80°C or to an imaging screen for 2 hours. Signal was visualized with a Phosphor Imager SI™ optical scanner and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Human and murine archvillin cDNAs

Human archvillin

Oligonucleotide primers (H96-2R, HU5P-GAP) (Table 1) were designed from human supervillin cDNA sequences (AF051850, AF051851) and used with the Clontech AP1 primer and Marathon-Ready™ human skeletal muscle cDNA in 5′-RACE (rapid amplification of cDNA ends) reactions with Advantage KlenTaq polymerase (Clontech, Palo Alto, CA). Clones (HSK02, HSK03, HSK16, HSK21, HSK31, HSK43, HSK61 and HSK69) encoding the 5′-end of the archvillin cDNA were obtained by cloning into pGEM-T (Promega). Colonies were identified by screening with a randomly-primed ^{32}P-labeled fragment of the human supervillin sequence corresponding to nt –12 to 1015 (Pope et al., 1998).

The rest of the archvillin coding sequence was generated using sets of non-degenerate primers (HSKP-1F and HSKP-1R; HSKP-2F and HSKP-2R) (Table 1) designed from human supervillin sequences and from the 5′-RACE archvillin products, above. These primers were used with the Marathon-Ready™ human skeletal muscle cDNA (Clontech) and Expand™ long-template polymerase (Roche Molecular Biochemicals, Indianapolis, IN) in touchdown thermal reactions to generate clones containing full-length human archvillin (FAV), or a smaller clone (SAV). FAV was completely sequenced in both directions, and SAV was completely sequenced in one direction and in both directions in the regions of muscle-specific sequence. An additional 419 bp of 3′-untranslated region (UTR) sequence was identified as the consensus of expressed sequence tags (ESTs) AA136154, AA149325, Z28958, AA442438, AW020040 and AA442798.

Murine archvillin

Murine archvillin cDNA also was cloned by PCR-RACE. The upstream primer (MSV-F1) (Table 1) corresponded to a consensus sequence from human and bovine supervillin 5′-UTRs (accession nos AF051850, AF025996) and rat EST AI549127. The reverse primer (MSV-R1) was designed using Primer Premier (Premier Biosoft International, Palo Alto, CA) from a consensus of 41 mouse ESTs homologous to the 3′-ends of the human and bovine supervillin coding sequences. Additional upstream sequences were obtained in nested 5′-

RACE reactions with the Clontech AP1 and AP2 primers and reverse primers complementary to nt 281-312 (MAV-R296) and nt 121-144 (MAV-R121) within the murine archvillin coding sequence, respectively. A forward (CRATY-F) primer for 3′-RACE with the Clontech AP1 primer was chosen from mouse EST #AA048260, which encodes a sequence homologous to the highly conserved amino acids 1089-1184 of bovine supervillin and amino acids 1085-1180 of human supervillin. Marathon-Ready™ skeletal muscle cDNA (Clontech) and the Herculase™ polymerase blend (Stratagene, La Jolla, CA) were included in touchdown thermal reactions to generate clones containing nearly full-length murine archvillin (clones M02, M03, M08), 5′-RACE products (clones M04U, M08U, M09U, M15U, M20U) and 3′-RACE products (clones M09, M23, M26, M23B).

Enhanced green fluorescent protein (EGFP)-tagged murine archvillin

The full-length coding region of the murine archvillin cDNA was generated by assembling consensus-matching regions of clones M02 and M09. This construct was then used as a template to generate by PCR cDNAs from forward primers with a 5′ *Xho*I site and reverse primers with a 5′ *Sac*II site (Table 1). PCR products were generated using Herculase™ polymerase and ligated into TOPO-TA vectors (Invitrogen, Carlsbad, CA), sequenced, recovered by digestion with *Xho*I and *Sac*II, and subcloned into pEGFP-C1 (Clontech). The expression of the resulting pEGFP-MAV chimeras was confirmed by western blotting of lysates from transfected C2C12 cells using α-H340 and an antibody against GFP (Roche Molecular Biochemicals, Indianapolis, IN).

DNA sequencing and structural analyses

All clones except SAV were fully sequenced in both directions by primer walking at the Iowa State University DNA Sequencing and Synthesis Facility (Ames, IA) or at the University of Massachusetts DNA Sequencing Facility (Worcester, MA). Consensus cDNA sequences for human and murine archvillins were constructed using Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, MI) and deposited in GenBank (accession nos AF109135 and AF317422, respectively). The sequence of the potential alternatively spliced murine archvillin is available as AF317423. Optimized sequence alignments with CLUSTALW 1.8 (Jeanmougin et al., 1998) were performed at http://clustalw.genome.ad.jp/. Protein compositional analyses were carried out using Web sites available at http://www3.ncbi.nlm.nih.gov/, http://www.up.univ-mrs.fr/~wabim/d_abim/compo-p.html, http://us.expasy.org/cgi-bin/protscale.pl, http://www.cbs.dtu.dk/services/Netphos/, and http://psort.nibb.ac.jp/.

Human multiple tissue northern

A human multiple tissue northern blot of poly(A)$^+$ RNA (Clontech) was hybridized overnight at 65°C in 7% SDS, 0.25 M Na$_2$PO$_4$, 10 mM EDTA, pH 7.3 (Church and Gilbert, 1984), with a ^{32}P-labeled random-primed probe prepared from a 697-bp *Xho*I/*Sma*I fragment corresponding to nt 4382-5079 from the human consensus coding region. The blot was washed three times at 65°C for 20 minutes in 2X SSC (0.3 M NaCl, 30 mM sodium citrate), 1.0% SDS, and exposed to film. The blot was stripped for reprobing by boiling for 10 minutes in 0.5% SDS in RNase-free water and then probed as above with a 187 bp *Xba*I fragment from the archvillin-specific region of the HSK61 clone (nt 1227-1414). Finally, the blot was probed with a ^{32}P-labeled random-primed β-actin control cDNA, washed as above, and washed twice more at 68°C for 30 minutes in 0.1X SSC, 0.1% SDS.

In situ hybridization

Detection of RNA from supervillin and/or archvillin genes was performed using nick-translated human supervillin cDNA from clone H09 (Pope et al., 1998). Detection of RNA from the β-cardiac myosin heavy chain gene (cMyHC) was performed using clone HM-1, a ~32 kb β-cMyHC specific genomic probe obtained from Choong-Chin Liew (University of Toronto, Ontario, Canada) (Yamauchi-Takihara et al., 1989). The methods used here, including procedures for non-isotopic probe preparation and fluorescence in situ hybridization, have been published in detail (Carter et al., 1991; Johnson et al., 1991). Images were captured using a Photometrics P-250 cooled CCD camera and the MetaMorph (Universal Imaging Corp., West Chester, PA) image-processing package. The microscope was a Zeiss Axioplan with a 100× Plan-Apo 1.4 objective and a triple band-pass filter set (63000, Chroma, Brattleboro, VT).

Immunofluorescence microscopy

Hamster thigh muscles were the generous gift of Thomas Schoenfeld (University of Massachusetts Medical School, Worcester, MA). Muscle sections of 5-7 μm were cut at –20°C on a Microm HM 500 OM microtome cryostat (Carl Zeiss, Walldorf, Germany). Before fixation, cultured myogenic cells or cryosectioned hamster muscle were washed at room temperature in sterile Dulbecco's phosphate buffered saline (DPBS), immediately fixed in either 4% paraformaldehyde in PBS, pH 7.4, or in –20°C methanol for 10 minutes, rinsed three times for 15 minutes in PBS, and then blocked for 30 minutes in blocking solution (10% horse serum, 1% BSA, 0.02% sodium azide, PBS). Cells and muscles were stained overnight at 4°C with 10 μg/ml affinity-purified anti-H340 in blocking solution. Rabbit polyclonal antibody against non-muscle myosin II heavy chain (Biomedical Technologies, Stoughton, MA) was used at a dilution of 1:30. Primary monoclonal antibodies were diluted with blocking solution as follows: anti-dystrophin (Novocastra Lab, Burlingame, CA), 1:20; anti-lamin A/C (Novocastra Lab, Burlingame, CA), 1:25; and anti-vinculin (Sigma Chemical Co.), 1:200. Nuclear DNA was visualized with ethidium homodimer-1 (Molecular Probes, Inc., Eugene, OR). F-actin was visualized with Alexa 594™-phalloidin (Molecular Probes). Samples were incubated for 1 hour at room temperature with a 1:2000 dilution of the appropriate secondary antibody (goat anti-rabbit Alexa 488™ or goat anti-mouse Alexa 594™; Molecular Probes), washed three times for 15 minutes in PBS, mounted in a Vecta mounting medium (Vector Laboratories, Burlingame, CA) and sealed with nail polish. Slides were analyzed on a Zeiss Axioskop fluorescence microscope or a Bio-Rad MRC 1024 laser scanning confocal microscope (Bio-Rad Laboratories, Hercules, CA) equipped with LaserSharp Version 3.2 software.

Results

Supervillin-related protein in muscle and myogenic cells

A ~250 kDa protein characteristic of mammalian muscle and myogenic cell lines was identified using two affinity-purified polyclonal antibodies specific for different sequences in the ~205 kDa non-muscle supervillin protein (Fig. 1A,B). The α-H340 antibody, directed against the first 340 residues of human supervillin, specifically recognizes supervillin in bovine neutrophils (Nebl et al., 2002). This antibody also recognized supervillin (205 kDa) in HeLa cells (Fig. 1A, lane 1, asterisk) and a larger, ~250 kDa polypeptide in human 50MB-1 myoblasts (Fig. 1A, lane 2) and in hamster (Fig. 1A, lane 3) and rabbit (Fig. 1B, lane 1) striated muscle. Specificity of the α-H340 antibody for supervillin-related sequences in muscle was shown both by immunoblot analyses (Fig. 1A,B) and in immunofluorescence micrographs of human (Fig. 1C) and murine (data not shown) myogenic cells. The α-pepA antibody, which was previously shown to be specific for amino acids
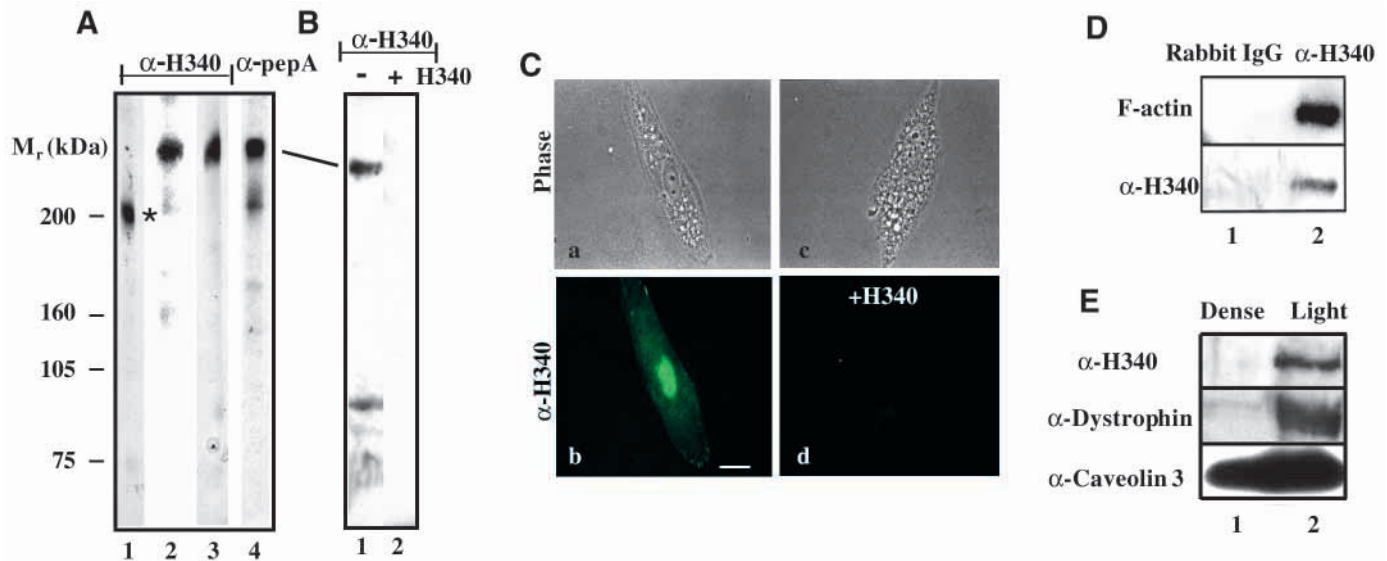
Fig. 1. Muscle contains a ~250 kDa F-actin binding protein that is related to supervillin. (A) Two antibodies (α-H340, α-pepA) against human supervillin (asterisk) recognize a larger protein in muscle cells (line). Immunoblots of human cervical carcinoma (HeLa S3) cells (lane 1), human 50MB-1 myoblasts (lane 2), hamster skeletal muscle (lane 3) and rabbit skeletal muscle (lane 4) were probed with affinity-purified rabbit polyclonal α-H340 (lanes 1-3) or α-pepA (lane 4) antibodies. Each lane of the 5% polyacrylamide SDS-gel was loaded with 100 μg total protein. Supervillin is a ~205 kDa polypeptide in non-muscle cells (lane 1, asterisk). (B) Specificity of the α-H340 antibody on immunoblots. Affinity-purified α-H340 antibody (0.5 μg/ml) was pre-incubated for 1 hour at 0°C without (–) or with (+) bacterially expressed H340 protein (60 μg/ml) before incubation with blot strips containing rabbit skeletal muscle (100 μg/lane) and visualization by ECL. Staining of both the major ~250 kDa polypeptide (line) and a less-prominent ~95 kDa band are competed by immunogen. Progressive loss of the larger band with time suggests proteolytic degradation. (C) Specificity of the α-H340 antibody in immunofluorescence. Phase images (a,c) and indirect immunofluorescence micrographs (b,d) of proliferating 50MB-1 cells stained with affinity-purified α-H340 and secondary antibodies. To show specificity, the α-H340 antibody used for C and D was pre-incubated with 20 μg/ml of the H340 immunogen for 1 hour before use. Bar, 5 μm. (D) Direct binding of ³²P-labeled F-actin to the ~250 kDa supervillin-like protein from mouse muscle. Immunoprecipitation with rabbit IgG as a negative control (lane 1) and with α-H340 (lane 2). The polypeptide specifically immunoprecipitated by α-H340 IgG binds both ³²P-labeled F-actin (top panel) and α-H340 antibody (lower panel). (E) Co-fractionation of the ~250 kDa supervillin-like protein with dystrophin and caveolin-3 in the crude plasma membrane fraction from rabbit skeletal muscle (Ohlendieck et al., 1991). Immunoblots with antibodies against H340, dystrophin and caveolin-3 in a higher-density membrane fraction enriched in T-tubules and sarcoplasmic reticulum (lane 1, Dense) and in the low-density membrane fraction enriched in sarcolemmal membranes (lane 2, Light) are shown.

900-918 in bovine supervillin (Pestonjamasp et al., 1997), also recognized a ~250 kDa polypeptide in rabbit skeletal muscle (Fig. 1A, lane 4), 50MB-1 myoblasts (data not shown) and C2C12 myoblasts (data not shown). A similarly sized, immunocrossreactive polypeptide in SDS-solubilized murine leg muscle was specifically immunoprecipitated with α-H340 antibody (Fig. 1D). After electrophoresis on SDS-polyacrylamide gels and electrotransfer to nitrocellulose, the immunoprecipitated polypeptide bound directly to α-H340 antibody and to radiolabeled F-actin (Fig. 1D, lane 2). Finally, the cross-reactive muscle protein co-fractionated with the sarcolemmal proteins, dystrophin and caveolin-3, as part of a low-density plasma membrane fraction from rabbit skeletal muscle (Fig. 1E, light fraction). These results show that the muscle protein, which is consistently larger than supervillin in several species and throughout myogenic differentiation, resembles non-muscle supervillin in that these proteins contain at least two epitopes in common, bind F-actin on blot overlays and are associated with plasma membranes.

Cloning of the muscle-specific supervillin isoform
To determine the molecular nature of the muscle protein and

the extent of its conservation across species, we used PCR-based strategies to clone supervillin-related cDNAs from human and murine skeletal muscle (accession nos AF109135 and AF317422, respectively). Consensus cDNA sequences of 7586 bp and 8138 bp were assembled from multiple human and mouse clones, respectively (Fig. 2A). BLASTN 2.1.1 searches of the dbest database (Altschul et al., 1997) suggested the presence of an additional 419 nt in the 3′- untranslated region (UTR) of the human sequence, indicating that the mRNAs are each ~8 kb long, exclusive of a poly(A) tail. The human cDNA is almost identical to that deduced from human genomic sequences (XM_030478), except for single basepair differences at position 3 within codons and changes at nucleotides 1319, 2017 and 4456, which would mostly result in conservative amino acid changes (Ala-189→Val, Ile-422→Val, Ala-1233→Pro). Thus, these differences may represent polymorphisms. The 3740 nt at the 3′-end of the cloned murine cDNA is virtually identical to a partial cDNA predicted from murine genomic sequences (XM_128880), with only three divergent base pairs in noncoding sequence.

Northern blots from several human tissues confirmed the presence of an abundant larger message in muscle (Fig. 2B-D). The ~7.5-kb message encoding supervillin (SV) was readily

Fig. 2. Cloning of muscle supervillin (archvillin) and northern analyses. (A) Diagram showing the coding regions (boxes), the 5′- and 3′-UTRs, and the sequences used as an archvillin-specific probe (probe 1) and as a probe for both archvillin and supervillin messages (probe 2). Lines represent partial sequences derived from gene-specific primers (no arrows) and 5′- (left arrows) and 3′- (right arrows) RACE products from skeletal muscle cDNAs. Archvillin-specific sequences in the 5′-halves of the coding regions (gold), predicted nuclear targeting signals (blue), and coding sequences for the highly conserved villin/gelsolin homology regions at the 3′-ends of the coding regions (pink) are indicated. Asterisks on clones M03 and M08 denote the location of an additional 23 bp of sequence. Consensus sequences of these human and murine cDNAs are available (accession nos AF109135, AF317422), as is the potential alternatively spliced murine cDNA sequence (AF317423). (B) Multiple tissue northern blot (Clontech) containing 2 μg per lane of poly(A)$^+$ RNA from heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7) and pancreas (lane 8) after hybridization with ^{32}P-labeled probe 2. The blot was exposed to XAR/MS film at –80°C with an intensifying screen for 24 hours. (C) Lanes from the same northern blot containing heart (lane 1) and skeletal muscle RNA (lane 6) after exposure to film at –80°C for only 3 hours. (D) The same northern blot after stripping and re-probing with archvillin-specific probe 1. (E) Hybridization with β-actin probe to show equal RNA loading. Migration positions of archvillin message are shown (AV, asterisks), as are the positions of supervillin mRNA (SV, arrowheads).
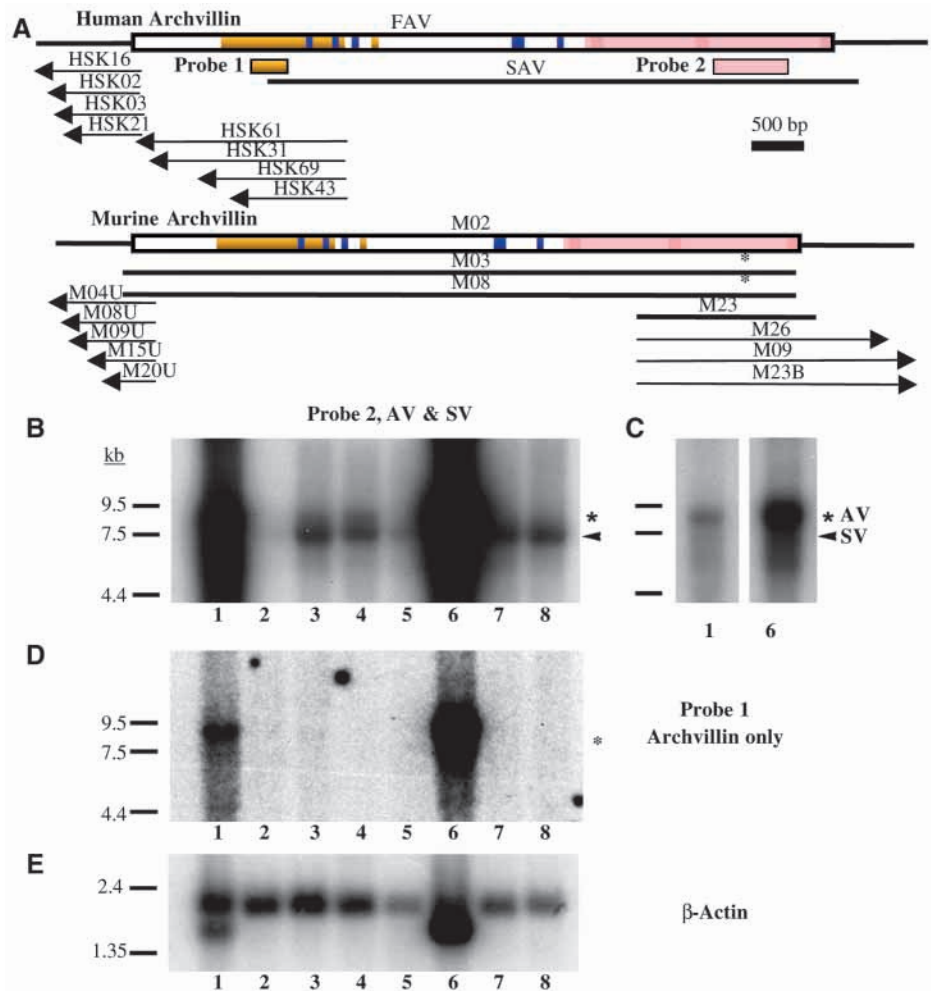
detected in placenta, lung, kidney and pancreas (Fig. 2B, lanes 3, 4, 7 and 8, arrowhead) and was present at lower levels in brain and liver (Fig. 2B, lanes 2 and 5). As predicted from previous northern dot blot analyses (Pope et al., 1998), cross-hybridizing mRNAs were most abundant in cardiac and skeletal muscle (Fig. 2B, lanes 1 and 6). Shorter exposures to film showed that the human muscle mRNAs were ~8.5 kb (Fig. 2C, asterisk), a size consistent with a 6645 bp coding region, 756 bp 5′-UTR, 903 bp 3′-UTR, and a ~200 bp poly(A) tail. Hybridization with a probe against sequences present only in the skeletal muscle cDNA (Fig. 2A, probe 1; see below) showed that the ~8.5 kb message was essentially absent from the non-muscle tissues analyzed (Fig. 2D, asterisk). Hybridization with β-actin sequences served as a control for mRNA loading and integrity (Fig. 2E).

Predicted proteins and message structure

The consensus human and mouse cDNA sequences were predicted to encode homologous proteins of 2214 and 2170 amino acids, respectively (Fig. 3). The human muscle protein was predicted to exhibit a molecular mass of 247,706.29 Da and an isoelectric point (pI) of 6.55; the mouse protein was predicted to be 243,161.63 Da with a pI of 6.44. Overall, these proteins were 80.7% identical and 90.2% similar, with the highest homology (97%) in the C-termini. Most sequence predicted for the human muscle protein was virtually identical to sequences in human (accession no. AF051850) and bovine (accession no. AF025996) supervillins. In particular, the villin-gelsolin homology region and sequences in the central part of the protein that have been shown to promote targeting of EGFP-chimeras into Triton-resistant nuclear aggregates (Wulfkuhle et al., 1999) were present in the muscle cDNA. This nuclear localization was apparently mediated by nuclear localization sequences (Fig. 3A, dark boxes) and a predicted coiled-coil sequence (Fig. 3A, hatched box). All of these structural features were found across species and in both muscle and non-muscle proteins.

In addition to sequences found in supervillin, three conserved muscle-specific sequences were identified. These sequences were encoded by exons that appeared to be differentially expressed in muscle. Two of the muscle-specific insert sequences altered the nature of the N-termini of the muscle proteins (Fig. 3A, boxed sequences). The first conserved insert sequence of 394 (human) or 372 (murine) residues is encoded by three muscle-specific exons on human
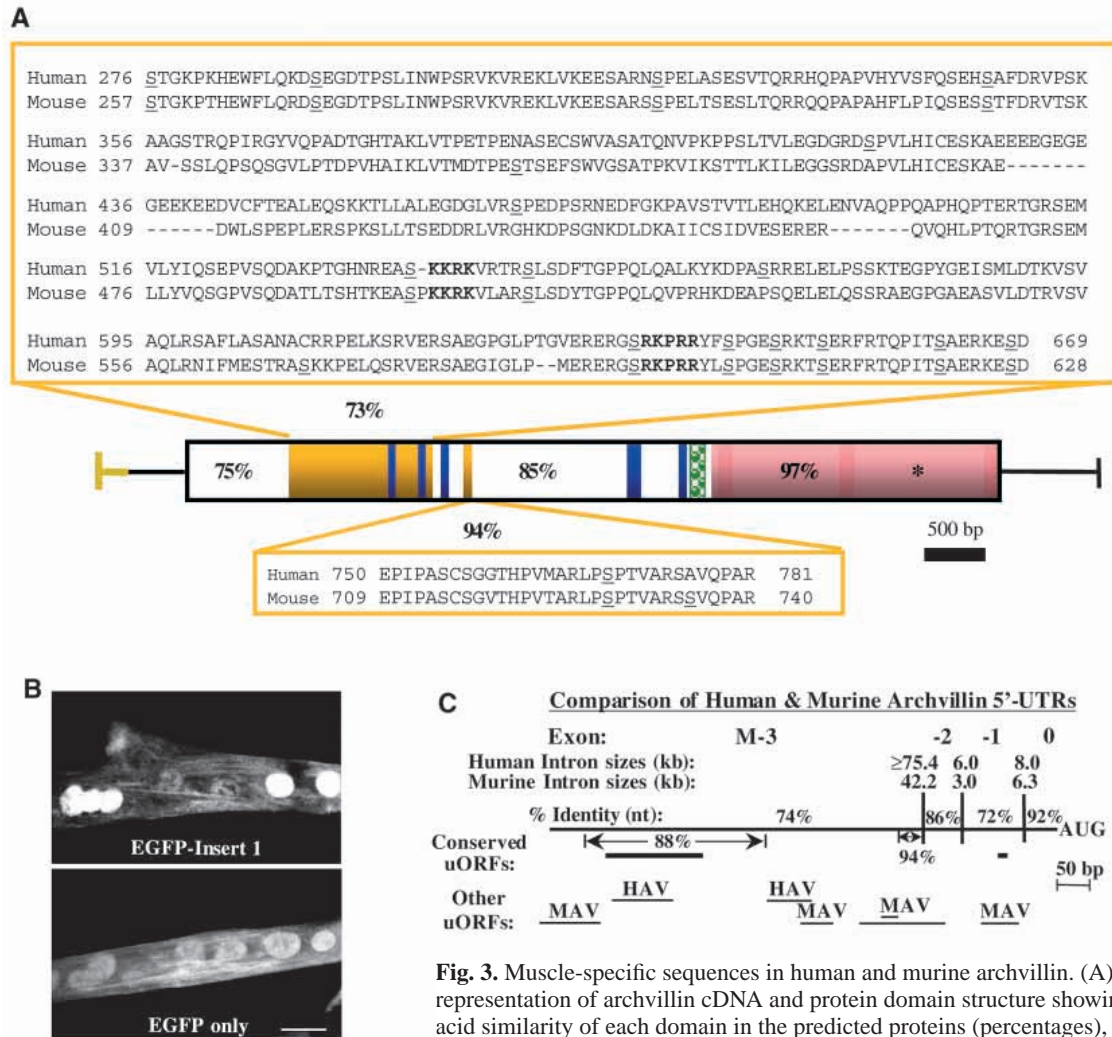
A

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Human 276 STGKPKHEWFLQKDSEGDTPSLINWPSRVKVREKLVKEESARNSPELASESVTQRRHQPAPVHYVSFQSEHSAFDRVPSK
Mouse 257 STGKPTHEWFLQRDSEGDTPSLINWPSRVKVREKLVKEESARSSPELTSESLTQRRQQPAPAHFLPIQSESSTFDRVTSK

Human 356 AAGSTRQPIRGYVQPADTGHTAKLVTPETPENASECSWVASATQNVPKPPSLTVLEGDGRDSPVLHICESKAEEEEGEGE
Mouse 337 AV-SSLQPSQSGVLPTDPVHAIKLVTMDTPESTSEFSWVGSATPKVIKSTTLKILEGGSRDAPVLHICESKAE-------

Human 436 GEEKEEDVCFTEALEQSKKTLLALEGDGLVRSPEDPSRNEDFGKPAVSTVTLEHQKELENVAQPPQAPHQPTERTGRSEM
Mouse 409 ------DWLSPEPLERSPKSLLTSEDDRLVRGHKDPSGNKDLDKAIICSIDVESERER------QVQHLPTQRTGRSEM

Human 516 VLYIQSEPVSQDAKPTGHNREASKKRKVRTRSLSDFTGPPQLQALKYKDPASRRELELPSSKTEGPYGEISMLDTKVSV
Mouse 476 LLYVQSGPVSQDATLTSHTKEASPKKRKVLARSLSDYTGPPQLQVPRHKDEAPSQELELQSSRAEGPGAEASVLDTRVSV

Human 595 AQLRSAFLASANACRRPELKSRVERSAEGPGLPTGVERERGSRKPRRYFSPGESRKTSERFRTQPITSAERKESD 669
Mouse 556 AQLRNIFMESTRASKKPELQSRVERSAEGIGLP--MERERGSRKPRRYLSPGESRKTSERFRTQPITSAERKESD 628
```

73%

75%   85%   97%   *

500 bp

94%

```
Human 750 EPIPASCSGGTHPVMARLPSPTVARSAVQPAR 781
Mouse 709 EPIPASCSGVTHPVTARLPSPTVARSSVQPAR 740
```

B

EGFP-Insert 1

EGFP only

C   Comparison of Human & Murine Archvillin 5'-UTRs

Exon: M-3   -2   -1   0
Human Intron sizes (kb):   ≥75.4 6.0 8.0
Murine Intron sizes (kb):   42.2   3.0 6.3
% Identity (nt):   74%   86% 72% 92% AUG
Conserved uORFs: ├── 88% ──┤   94%   50 bp
Other uORFs: MAV   HAV   HAV   MAV   MAV   MAV

Fig. 3. Muscle-specific sequences in human and murine archvillin. (A) Schematic representation of archvillin cDNA and protein domain structure showing the amino acid similarity of each domain in the predicted proteins (percentages), the amino acid sequences of the two muscle-specific coding inserts, the five predicted nuclear targeting sequences (blue bars), the predicted coiled-coil domain (green patterned box) and the location of a 23 bp insert sequence found in clones M03 and M08 (asterisk). Gold shading denotes the relative extents of the two N-terminal muscle-specific inserts and the highly conserved C-terminal villin/gelsolin homology regions. If stable protein can be produced from clones M03 and M08, the C-terminal sequence after Q-1948 in mouse archvillin would be altered to ALFSFLWKILEVLTSRPACSSSAAPPETSLRQSLCTPHKRPLPSAPCLSCRRTCTAR-RSQLSSLLTTITRCTSGKAGGPLKTR, resulting in a smaller, more basic protein (~227 kDa, pI=8.16) without the villin-like headpiece. (B) Fluorescence localization in C2C12 myotubes expressing a chimera of EGFP and murine archvillin insert 1 sequence (MAV 257-629, left) or EGFP alone (right) is consistent with the prediction that muscle insert 1 contains functional nuclear targeting sequences. Bar, 5 μm. (C) The muscle-specific upstream exon M-3 contains regions of high sequence similarity. Regions of 254 nt and 34 nt in exon M-3 that exhibit 88% and 94% identity, respectively, between human and mouse archvillin cDNAs are shown (arrows). Intron locations are denoted schematically by thick vertical bars, and intron sizes are shown. Exons –2 and –1 are present in both muscle and non-muscle cDNAs. Exon M-3 is consistently observed in muscle-specific cDNAs and in ESTs from muscle-rich tissues. Exon nomenclature was based on the location of the initiator AUG because the large size of the first muscle-specific intron and the growing number of ESTs with homology to other upstream genomic sequences suggest the potential for many other exons encoding supervillin/archvillin 5′-UTR sequences. Upstream open reading frames (uORFs), two of which are conserved across species (thick bars) are present in human (HAV) and murine (MAV) archvillin 5′-UTRs.

chromosome 10 (NT_008609) and murine chromosome 18 (NW_000134). The human and murine amino acid sequences are 63% identical and 73% similar to each other, overall, with regions of sequence conservation that are ≥90% identical. The first muscle-specific inserts of the human and murine proteins were predicted to contain two conserved nuclear targeting sequences (Fig. 3A, bold type) and to be enriched in arginine, glutamic acid, proline and serine residues. Expression of insert 1 as a chimeric protein with EGFP greatly enhanced the amount of fluorescent EGFP in the nucleus (Fig. 3B),

supporting its predicted nuclear targeting capability. In both human and mouse, the second muscle-specific insert of 32 amino acids was separated from the first by 79 residues encoded by three exons also expressed in non-muscle supervillin. The second muscle-specific inserts were 90% identical and 94% similar between species and were relatively rich in arginine, proline and serine residues. Consequently, several sites fitting consensus sequences for serine/threonine protein kinases were predicted within each insert (Fig. 3A, underlines). Taken together, the two inserts encode muscle-
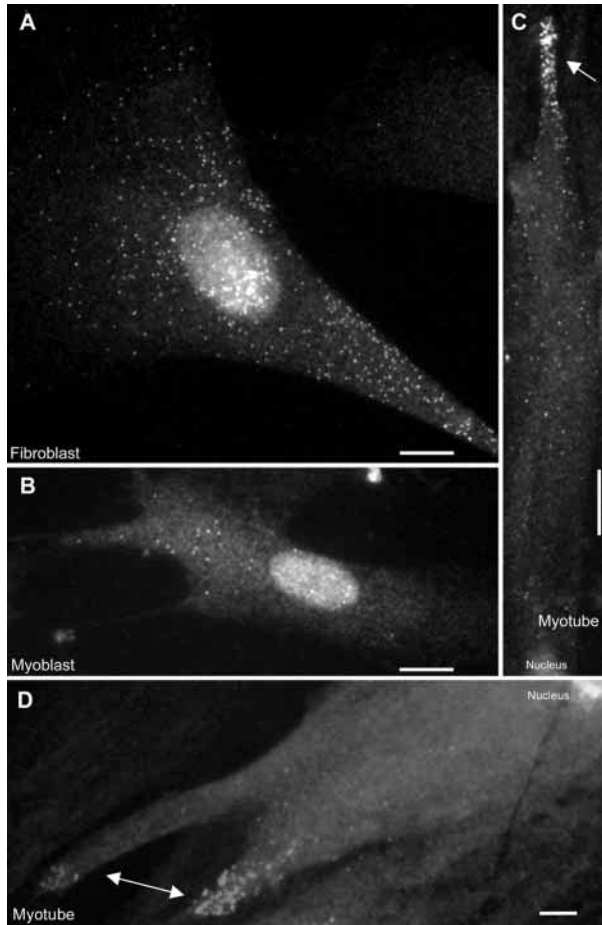
Fig. 4. Endogenous archvillin mRNA is clustered at the ends of myotubes, but no clustering is observed for supervillin mRNA in polarized human fibroblasts or for archvillin mRNA in myoblasts. (A) WI-38 fibroblasts, (B) 50MB-1 myoblasts and (C,D) 50MB-1 myotubes were fixed, probed with digoxigenin-labeled sequences conserved in human supervillin and archvillin mRNAs, and visualized with rhodamine-labeled anti-digoxigenin. Note the strong granular staining at the tips of myotubes (arrows); the most proximal of the centrally localized nuclei is indicated (nucleus). Bars, 5 μm.

Fig. 5. Archvillin mRNA localizes to the tips of myotubes, in contrast to the more central distribution observed for mRNA encoding the β-cardiac myosin heavy chain. Double labeling of a human 50MB-1 myotube with probes for (A) archvillin and (B) myosin heavy chain mRNAs. (C) The composite image shows archvillin (red), myosin heavy chain (green) and nuclear DNA (blue). Archvillin mRNA was hybridized using a cDNA probe labeled with digoxigenin-11-dUTP and detected with rhodamine-conjugated anti-digoxigenin; mRNA encoding the β-cardiac myosin heavy chain was hybridized with a nick-translated probe containing biotin-16-dUTP and detected with FITC-conjugated avidin. DNA was labeled with 4′,6-diamidino-2-phenylindole (DAPI). The most proximal of the centrally localized nuclei is at the left of the image. Bar, 5 μm.

specific sequences of 47.0 kDa (human) and 44.6 kDa (mouse) that are absent from non-muscle supervillin. To denote both the identical regions and the differences between the muscle and non-muscle proteins and to facilitate comparisons between the two, we propose to call the second, muscle-specific isoform of supervillin 'archvillin'.

The third conserved muscle-specific sequence (exon M-3 in Fig. 3C) represented the 5′-ends of leader sequences (5′-UTRs) of at least 756 nt and 770 nt in human and mouse archvillin cDNAs, respectively. Archvillin 5′-UTRs were much longer than the 20-100 nt leader sequences of most vertebrate mRNAs (Kozak, 1987) and contained six (human) or eight (murine) AUG codons upstream of the consensus start site. Despite their locations of ~90 kb (human) and ~52 kb (mouse) upstream of the first protein-coding exon, the human and murine M-3 exons were 74% identical to each other overall and contained sequences of 254 nt and 34 nt that were 88% and 94% identical across species (Fig. 3C, double arrows). The first conserved upstream open reading frame (uORF) in exon M-3 potentially
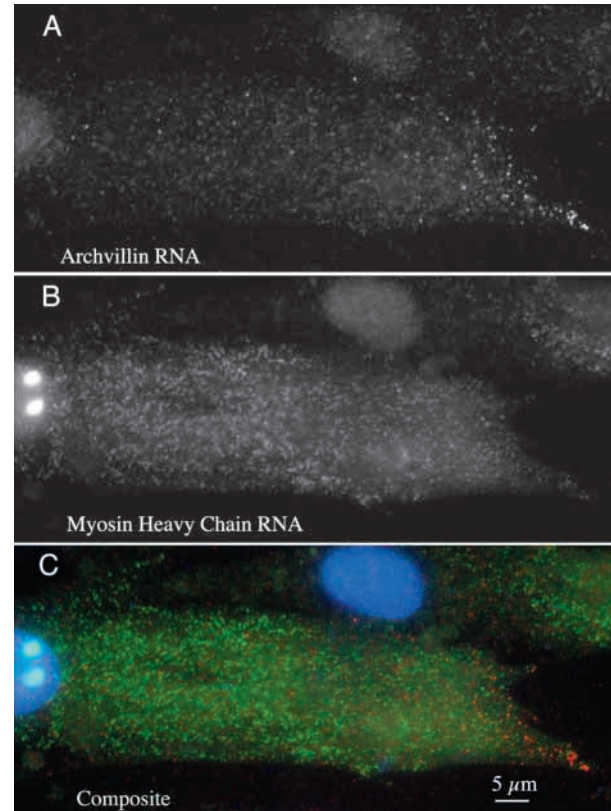
encoded a peptide of 49 residues; the second conserved uORF potentially encoded peptides of 4 (human, cow) or 17 (mouse) residues (Fig. 3C, thick bars). Other uORFs were present in both human (HAV) and murine (MAV) archvillin 5′-UTRs. Such sequences are found in only ~9% of all vertebrate mRNAs, but are present in two thirds of messages encoding oncogenes and mRNAs that are regulated post-transcriptionally near the site of protein synthesis (reviewed by Hellen and Sarnow, 2001; Kozak, 1987; Morris and Geballe, 2000).

The archvillin 3′-UTR was much less remarkable. As is true for most messages (Hawkins, 1988), the entire 3′-UTR sequence (Pope et al., 1998) is found within the last coding exon. All known supervillin and archvillin cDNAs and ESTs from both muscle and non-muscle sources contain this sequence, suggesting a lack of alternative splicing in the 3′-UTR. The 3′-UTR sequences diverged across species, except for a conserved sequence of 131-142 nt located ~302-317 nt downstream of the stop codon (not shown). This conserved 3′

Fig. 6. Archvillin in hamster skeletal muscle is primarily sarcolemmal, localizing at costameres and within peripheral nuclei. The α-H340 signal localizes predominantly as 'arches' in optical cross-sections (B,D, double arrowhead*s*) that resolve as alternating circumferential thick and thin bands with occasional longitudinal striations in confocal sections within the plane of the sarcolemma (C, inset, arrow). Archvillin colocalizes with costameric dystrophin in both transverse (D-F) and oblique (G-I) muscle sections. Colocalization with ethidium homodimer-1 in peripheral nuclei also is seen (J-L, arrows), as is a small amount of staining within the muscle cells (M-O, asterisks). Phase (A), epifluorescence (B) and confocal fluorescence sections (C-O) show the colocalization of archvillin (B,C,D,G,J,M) with anti-dystrophin (E,H) and ethidium homodimer-1 (K,N). Composite images (F,I,L,O) were generated by superimposition of the α-H340 signals in green and dystrophin (E,H) and ethidium homodimer-1 (K,N) signals in red; areas of overlap appear yellow or orange. Bars, 5 μm (C), 10 μm (A-B,G-I) and 20 μm (D-F,J-O).
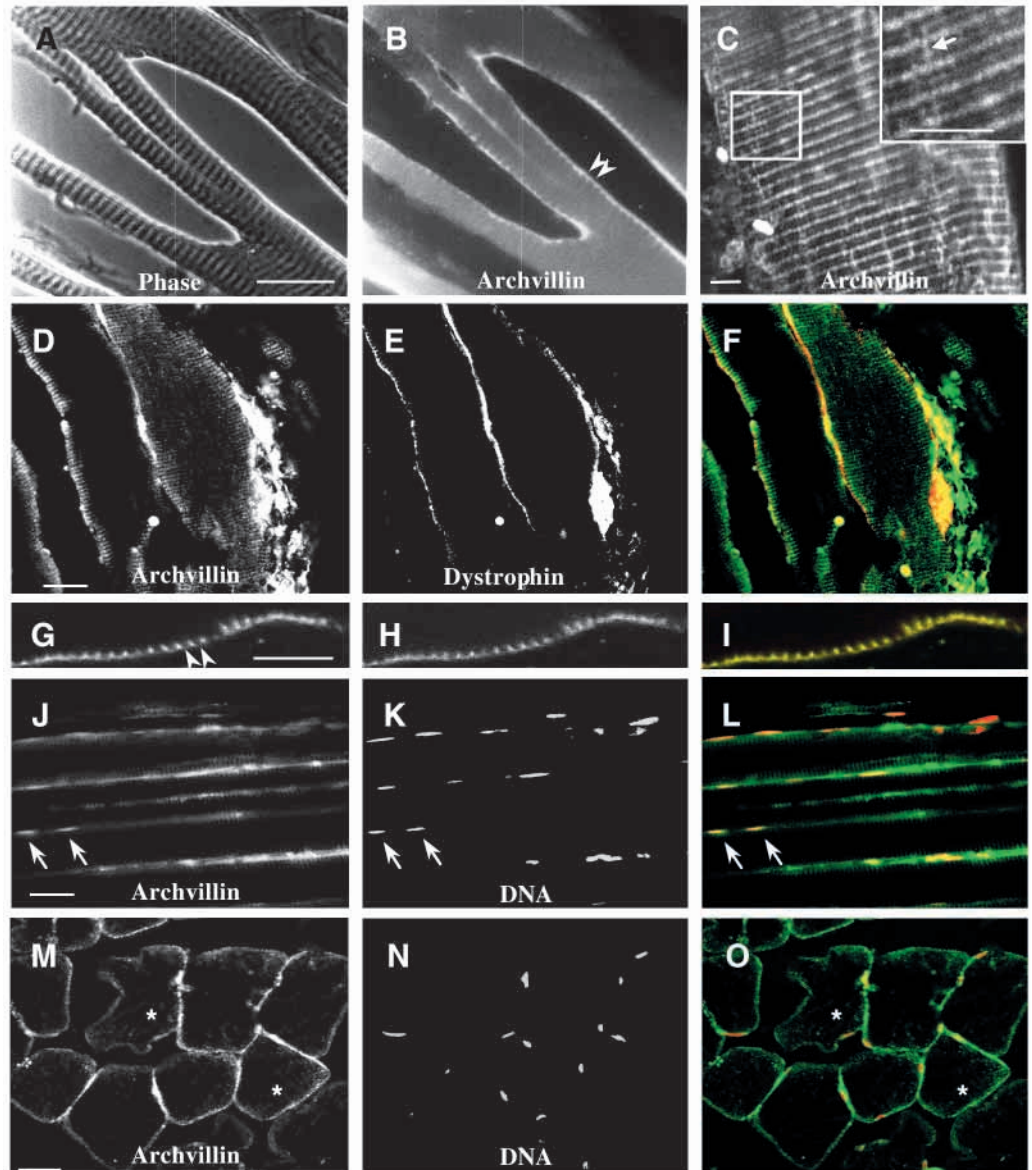
sequence contained motifs characteristic of binding sites for the ELAV/Hu family of message stabilizing and targeting proteins (reviewed by Antic and Keene, 1997; Brennan and Steitz, 2001; Keene, 2001). The presence of ELAV/Hu motifs suggested that archvillin and supervillin messages may be stabilized against degradation (reviewed by Jacobson and Peltz, 1996) and raised the possibility of mRNA targeting (Antic and Keene, 1998; Gao and Keene, 1996).

Message localization

To test the possibility that supervillin and/or archvillin messages can be targeted within the cytoplasm, we examined non-muscle and muscle cells by in situ hybridization with a nick-translated human cDNA probe to sequences found in both mRNAs (Fig. 4). Specificity of this probe for the single SVIL locus on chromosome 10 was shown previously (Pope et al., 1998). Sequences hybridizing with SVIL exons were found throughout the cytoplasm, as well as within the nuclei,

of migrating non-muscle WI-38 fibroblasts (Fig. 4A) and in the cytoplasm of elongated, but undifferentiated, 50MB-1 myoblasts (Fig. 4B). By contrast, prominent clusters of hybridizing species – presumably archvillin mRNAs because 50MB-1 cells do not express detectable amounts of supervillin (Fig. 1) – were observed within the elongated processes of differentiating myotubes (Fig. 4C,D, Fig. 5A). These clusters appeared as large granules in fluorescence optics.

Although significant amounts of diffusely distributed archvillin messages were also observed in myotube cytoplasm, most of the granular clusters were highly polarized, especially compared with messages for the myofibrillar protein, β-cardiac myosin heavy chain (Fig. 5). The myosin heavy chain mRNA exhibited a relatively uniform distribution throughout the myotube (Fig. 5B, green in Fig. 5C) when hybridized in the same experiment under conditions identical to those used for the archvillin mRNA (Fig. 5A, red in Fig. 5C). The more highly polarized localization of the archvillin message was
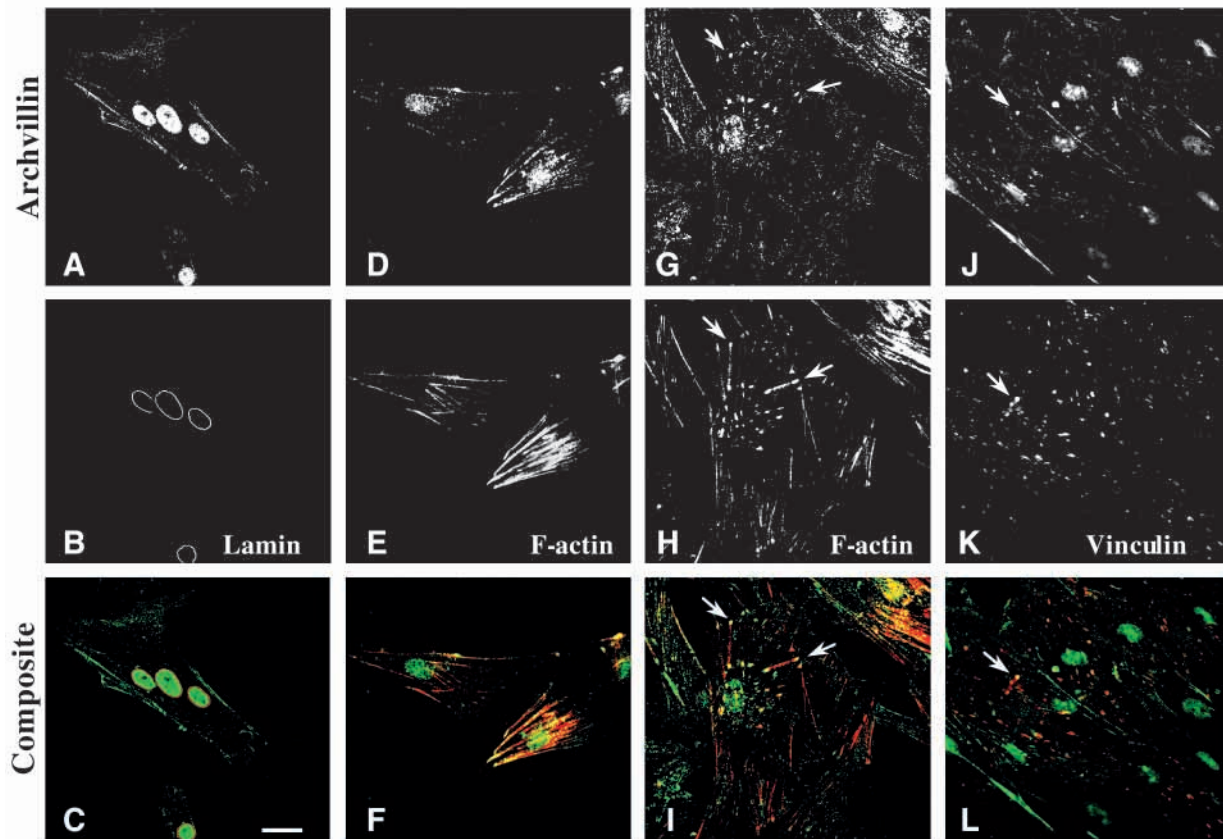
Fig. 7. Archvillin localizations in 50MB-1 myoblasts. Confocal sections of human 50MB-1 myoblasts showing α-H340 signals within nuclei (A-C), in association with actin filament bundles near the basal plasma membrane (D-I) and as punctae on basal membrane surfaces (J-L). Double-label immunofluorescence of archvillin (A,D,G,J) with anti-lamin A/C (B), Alexa™ 594-labeled phalloidin (E,H) and anti-vinculin (K). Composite images (C,F,I,L) were generated by superimposition of the α-H340 signals in green and lamin (C), F-actin (F,I) and vinculin (L) signals in red; areas of overlap appear yellow. Arrows denote localizations at the ends of actin filaments (G-I) and occasional sites of colocalization with vinculin at the basal membrane (J-L). Bar, 20 μm.

independent of the label used, with similar results observed in experiments in which the labels for the two probes were reversed (data not shown). Thus, the enrichment of hybridization signal for archvillin mRNA at the tips of myotubes was specific for this message and was not an artifact of fixation, permeability or some other technical condition. Given that similar clusters of RNA-containing granules are characteristic of translocated mRNP complexes in oocytes, budding yeast and neurons (reviewed by Mohr and Richter, 2001), our results are consistent with increased mRNP granule formation and/or polarized targeting of archvillin messages during myoblast differentiation and fusion into myotubes.

Archvillin protein localization in muscle

Archvillin was predominantly localized at the cell peripheries in dissected skeletal muscle (Fig. 6). In optical and oblique cross-sections obtained by epifluorescence (Fig. 6B) and confocal (Fig. 6G) microscopy with affinity-purified α-H340 antibody, archvillin appeared as 'arches' along the sarcolemma (Fig. 6, double arrowheads). These structures were revealed as alternating thick and thin bands in en face confocal sections (Fig. 6C), with occasional longitudinal strands (arrow in Fig. 6C, inset). This appearance is characteristic of the myofibril-to-

sarcolemmal attachment sites called costameres (Craig and Pardo, 1983; Pardo et al., 1983; Williams and Bloch, 1999a). In fact, the 'arched' α-H340 signal colocalized with the costameric protein dystrophin at the sarcolemma (Fig. 6D-F,G-I).

Enhanced α-H340 signal was also observed in larger structures at the myofiber periphery (Fig. 6J,M; green in Fig. 6L,O). These larger peripheral signals often colocalized with the DNA marker, ethidium homodimer-1 (Fig. 6K,N; red in Fig. 6L,O; overlap in yellow/orange), suggesting that archvillin is also associated with peripherally located myonuclei and/or the nuclei of satellite cells. Such presumptive nuclear localizations were more easily seen in longitudinally sectioned muscle (Fig. 6J-L, arrows) than within cross-sections (Fig. 6M-O). Additional minor α-H340 staining within the interiors of the muscle cells (Fig. 6M,O, asterisks) was difficult to localize, but preliminary observations suggested that small amounts of archvillin may also be found in the vicinity of t-tubules and/or the spaces between the Z-bands of adjacent myofibrils (not shown).

Early myogenesis

The apparent amounts and localizations of archvillin changed during differentiation along the myogenic pathway (Figs 7-9). In undifferentiated human 50MB-1 (Fig. 1C, Fig. 7) and
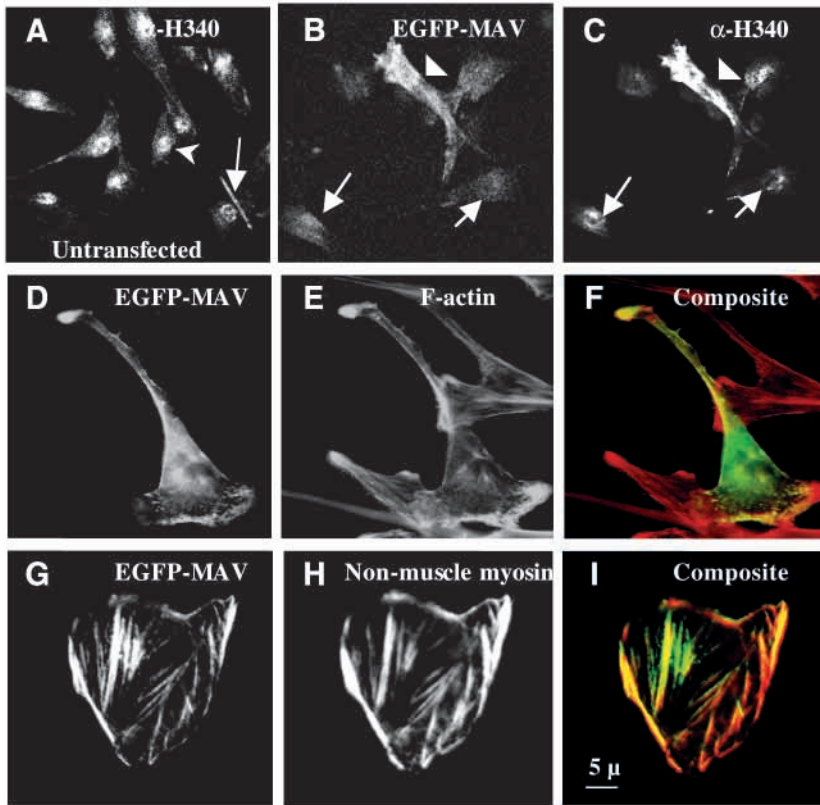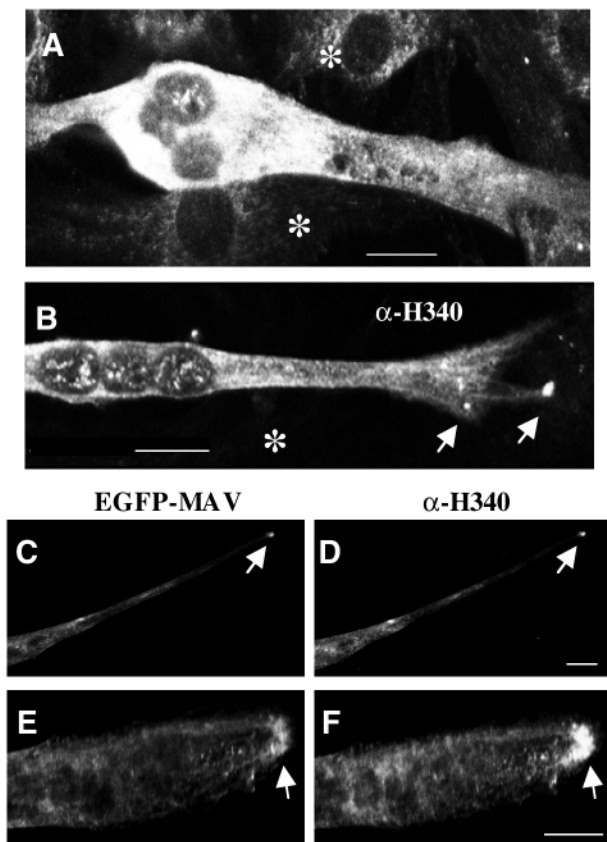
Fig. 8. EGFP-tagged full-length murine archvillin (MAV-FL) colocalizes with endogenous archvillin, F-actin and non-muscle myosin II. Confocal fluorescence images showing C2C12 myoblasts visualized for (A) endogenous archvillin localization with α-H340 in nuclei (arrowhead) and at the plasma membrane (arrow); (B,C) colocalization of EGFP and α-H340 signals in cells expressing low (arrows) and moderate (arrowhead) levels of EGFP-tagged archvillin; and colocalization of (D,G) EGFP-tagged full-length archvillin with (E) Alexa™-594 phalloidin-stained F-actin or (H) antibodies against non-muscle myosin II heavy chain. Composite images (F, I) were generated by superimposition of the α-H340 signals in green and phalloidin (E) or non-muscle myosin II (H) signals in red; areas of overlap appear yellow. Myoblasts in panels G-I were pre-extracted with 0.2% Triton X-100 (5 minutes, 0°C) to better visualize cytoskeletal structures. Bar, 5 μm.

murine C2C12 (Fig. 8) myoblasts, the α-H340 signal was largely nuclear with some cytoplasmic staining (Fig. 1C, Fig. 7A,D,G,J, Fig. 8A). The nuclear signal was punctate and distributed throughout the nucleoplasm, but was excluded from nucleoli (Fig. 7A-C, Fig. 8A). Cytoplasmic staining consisted of both diffuse and punctate signals, with preferential localization of immunoreactive punctae along the plasma membrane (Fig. 7D-L). Membrane-associated punctae often colocalized along the sides and at the ends of microfilament bundles (Fig. 7D-I, arrows). Partial colocalization of archvillin punctae with vinculin also was observed (Fig. 7J-L, arrows), suggesting occasional overlap with vinculin-containing focal contacts (Zamir and Beiger, 2001).

Myogenic cells expressing low levels of EGFP-tagged full-length murine archvillin exhibited a similar pattern of staining (Fig. 8). Signal in undifferentiated myoblasts was mostly nuclear (Fig. 8B,C, arrows), and the relative amount of cytoplasmic staining, as detected by both EGFP and the α-H340 antibody, increased in cells expressing higher levels of transfected protein (Fig. 8B,C, arrowheads). Confocal sections of basal membrane surfaces exhibited punctate and fibrillar archvillin distributions that colocalized well with F-actin (Fig. 8D-F) and non-muscle myosin II (Fig. 8G-I).

Endogenous levels of archvillin increased during differentiation into myotubes; essentially all of this increase was due to enhanced staining of cytoplasm and/or membrane structures (Fig. 9A,B). Polarized clusters of archvillin protein were observed both in untransfected C2C12 myotubes (Fig.

Fig. 9. Increased amounts and polarization of endogenous and EGFP-tagged murine archvillin in myotubes. Confocal images showing increased amounts of archvillin staining in untransfected myotubes (A,B), as compared with adjacent myocytes (asterisks). In addition to the overall increase in staining, large clusters of fluorescence are observed for endogenous archvillin (B) and for EGFP (C,E) and α-H340 signals (D,F) at the tips of myotubes expressing low levels of EGFP-tagged murine archvillin (EGFP-MAV). In both small (C,D) and large (E,F) myotubes, EGFP and α-H340 archvillin signals colocalize at myotube tips (arrows) and as punctae along the membrane and within the cell. Bars, 20 μm.
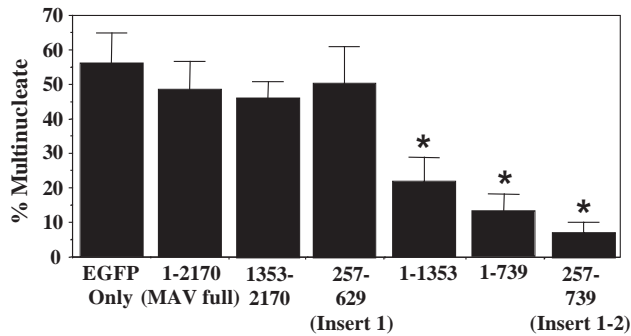
Fig. 10. Inhibition of myotube formation by EGFP-tagged murine archvillin sequences. EGFP-tagged proteins encoding the designated murine archvillin amino acids were transfected into C2C12 myoblasts, and the percentages of expressing cells that were multinucleate were scored after 6 days in differentiation medium. Column heights and error bars represent the means and standard deviations, respectively, of four separate experiments. Statistical significance was assessed with the Tukey-Kramer multiple comparisons test and by Student's *t*-tests. Both analyses indicated that C2C12 cells transfected with EGFP-tagged murine archvillin sequences 1-1353, 1-739 and 257-739 were significantly less likely to be present in myotubes than were cells similarly transfected with either the EGFP tag alone or with EGFP-tagged full-length murine archvillin (*$P<0.001$).

9A,B) and in myotubes transfected with low levels of EGFP-tagged archvillin (Fig. 9C-F). The polarized localization of EGFP-tagged archvillin from a construct lacking archvillin 5′- and 3′-UTR sequences indicated that these sequences were not required for protein targeting during early myogenesis. This distribution presaged the appearance of dystrophin at myotube tips because dystrophin is not expressed at appreciable levels until 10-11 days after the induction of differentiation (Belkin and Burridge, 1995; Kobayashi et al., 1995).

As an initial assay for functional involvement in myogenesis, C2C12 cells were transiently transfected with a series of murine archvillin deletion proteins that were tagged with EGFP at their N-termini. After six days in differentiation medium, the percentages of total fluorescent cells present as multinucleated myotubes were scored as a measure of the relative efficiency of differentiation (Fig. 10). By this method, about half of the cells in differentiation medium that were expressing either EGFP alone or EGFP-tagged full-length archvillin (amino acids 1-2170), archvillin C-terminus (aa 1353-2170) or the first coding muscle-specific archvillin sequence (aa 257-629, insert 1) were recovered as myotubes (Fig. 10), despite a range of transfection efficiencies (not shown). Because each myotube may contain nuclei from several transfected myoblasts, these percentages are probably underestimates of the overall differentiation efficiencies. By contrast, cells expressing either archvillin N-terminal sequences (aa 1-1353, aa 1-739) or the N-terminal region containing both muscle-specific coding sequences (aa 257-739) were far less efficient at forming myotubes under identical conditions in four separate experiments (Fig. 10). These results are consistent with a dominant-negative effect of archvillin N-terminal sequences during early myogenesis.

Discussion

In this study we have identified and cloned archvillin – a larger, differentially spliced isoform of supervillin. Archvillin is the isoform found in myogenic cell lines and in cardiac and skeletal muscles, which are the tissues in which supervillin/archvillin messages are the most abundant. The differences between supervillin and archvillin are conserved between mouse and human. These differences include an initial muscle-specific exon containing an extended 5′ leader sequence with potential post-transcriptional control elements and four exons encoding two muscle-specific inserts of 372-394 and 32 amino acids, respectively. The two conserved functional nuclear targeting signals in the first of these inserts may contribute to the targeting of archvillin to nuclei, a localization not observed for supervillin in differentiated cells.

Archvillin binds directly to F-actin, co-isolates with dystrophin and caveolin-3 in low-density sarcolemmal membranes, and colocalizes with dystrophin at costameres in skeletal muscle. In myoblasts, archvillin localizes primarily within nuclei but is also concentrated at the plasma membrane with F-actin, non-muscle myosin II and vinculin. A role for archvillin during early myogenesis is suggested by the striking localization of archvillin protein and message at the ends of differentiating myotubes and by the apparent dominant-negative inhibition of myotube formation on overexpression of chimeric proteins containing N-terminal archvillin sequences.

A logical hypothesis is that archvillin serves as an additional linkage between actin filaments and the plasma membrane at costameres. Although dystrophin is required for sarcolemmal integrity, loss of muscle function develops only over time, suggesting the existence of partially redundant proteins (Blake et al., 2002; Hoffman et al., 1987). One such protein is the dystrophin-related protein, utrophin, but even mice lacking both dystrophin and utrophin exhibit superficially normal muscle function until about four weeks of age (Grady et al., 1997). Other possible actin-membrane linkages at costameres include protein complexes that contain spectrin or focal adhesion proteins, including integrin, vinculin and α-actinin (Berthier and Blaineau, 1997; Stromer, 1995). In support of the idea of at least partial functional redundancy, costameres containing β-spectrin and vinculin are retained, although frequently disarranged, in sarcolemma of the *mdx* mouse (Williams and Bloch, 1999b). Conversely, loss of α- or β-spectrin in nematodes leads to muscle dysfunction (Hammarlund et al., 2000; Moorthy et al., 2000), suggesting cross-talk among muscle membrane skeleton proteins. Because archvillin contains all the sequences found in supervillin, a nonmuscle protein that interacts with vinculin-containing focal adhesions (Wulfkuhle et al., 1999) and co-isolates with spectrin, non-muscle myosin II and α-actinin (Nebl et al., 2002; Pestonjamasp et al., 1997), archvillin is likely to participate in the cross-talk between the spectrin- and focal adhesion-based membrane skeletons at costameres in muscle.

Similarities between signal transduction pathways and membrane-cytoskeletal attachments in muscle and non-muscle cells are reasonable in the context of our current understanding of myogenesis (Perry and Rudnick, 2000; Taylor, 2002). Myogenic cell migration from embryonic somites (Perry and Rudnick, 2000) and the recruitment of fusion-competent myoblasts to the vicinity of founder cells (Taylor, 2002) are morphologically reminiscent of

chemotactic behaviors exhibited by neutrophils and macrophages (reviewed by Weiner, 2002). Furthermore, both muscle and non-muscle cells must be able to regulate cytoskeletal organization and cell adhesion to the extracellular matrix during changes in tensile forces. However, there are clear differences in scale between muscle and non-muscle cells, and muscle-specific expression of protein isoforms is the rule, rather than the exception. Thus, both similarities and differences are observed in membrane skeleton proteins, such as archvillin and supervillin, that participate in the regulation of motile cell architecture.

Archvillin may also play a role within myonuclei during early myogenesis. This possibility is consistent with its strong nuclear immunolocalization before and during muscle differentiation and by the ability of supervillin sequences that are also present in archvillin to bind and transactivate the androgen receptor in prostate and muscle cells (Ting et al., 2002). Although a link between androgen receptor activation and increased muscle mass is widely recognized (Sheffield-Moore, 2000; Tindall, 2000), the mechanism is unknown. One possibility is enhanced expression of a regulatory factor, such as myogenin (Lee, 2002), that potentiates myoblast differentiation. But non-genotropic effects of androgens on membrane-associated signal transduction pathways are also recognized (Benten et al., 1999; Kousteni et al., 2001). Thus, interference with either type of signaling pathway could reasonably account for the apparent dominant-negative effects of EGFP-tagged archvillin N-terminal sequences.

Another possibility, suggested by the colocalization of archvillin with F-actin, vinculin and non-muscle myosin II at the membrane in myoblasts, is that archvillin acts in the early stages of muscle-specific membrane specializations. For instance, the first step during myofibril formation is the assembly of punctae containing F-actin, non-muscle myosin II and vinculin at the plasma membrane (LoRusso et al., 1997; Rhee et al., 1994; Sanger et al., 2000). During the maturation of these premyofibrils into contractile myofibrils, non-muscle myosin II is replaced by muscle-specific myosin II that assembles into thick filaments with the dimensions of muscle A-bands (LoRusso et al., 1997; Rhee et al., 1994).

Finally, the localizations of archvillin message and protein at the ends of myotubes suggest a contribution to the formation and/or stability of myotendinous junctions. These structures, which transduce longitudinal forces during muscle contraction, are enriched in several types of membrane skeletons, including dystrophin/dystroglycan- and integrin-based transmembrane attachments (Benjamin and Ralphs, 2000; Small et al., 1992; Tidball, 1991). The conserved uORFs in the long 5′ leader sequence of the targeted archvillin mRNA hint at the possibility that archvillin protein synthesis may be under local control at the ends of myotubes and/or myotendinous junctions. Post-transcriptional control of key regulatory proteins is well documented in oocytes and neurons (Mohr and Richter, 2001), and it is intriguing to note that the membrane skeleton composition and architecture at this important region of the sarcolemma may be capable of relatively rapid remodeling in response to local stimuli.

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