

Regulation of Microtubule Dynamics and Myogenic Differentiation by MURF, a Striated Muscle RING-Finger Protein

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Abstract. The RING-finger domain is a novel zinc-binding Cys-His protein motif found in a growing number of proteins involved in signal transduction, ubiquitination, gene transcription, differentiation, and morphogenesis. We describe a novel muscle-specific RING-finger protein (MURF) expressed specifically in cardiac and skeletal muscle cells throughout pre- and postnatal mouse development. MURF belongs to the RING-B-box-coiled-coil subclass of RING-finger proteins, characterized by an NH₂-terminal RING-finger followed by a zinc-finger domain (B-box) and a leucine-rich coiled-coil domain. Expression of MURF is required for skeletal myoblast differentiation and myotube fusion. The leucine-rich coiled-coil domain of

MURF mediates association with microtubules, whereas the RING-finger domain is required for microtubule stabilization and an additional region is required for homo-oligomerization. Expression of MURF establishes a cellular microtubule network that is resistant to microtubule depolymerization induced by alkaloids, cold and calcium. These results identify MURF as a myogenic regulator of the microtubule network of striated muscle cells and reveal a link between microtubule organization and myogenesis.

Key words: microtubule • RING-finger • cytoskeleton • striated muscle

Introduction

The RING-finger is an unusual type of Cys-His zinc-binding motif found in a growing number of proteins with roles in signal transduction, gene transcription, differentiation, E2-dependent ubiquitination, and morphogenesis (Borden, 1998; Freemont, 2000; Saurin et al., 1996). A RING-B-box-coiled-coil (RBCC)¹ subclass of RING-finger proteins contains an NH₂-terminal RING-finger followed by a single or multiple additional zinc-finger domains, termed B-boxes, and a leucine-rich coiled-coil domain (Borden, 1998). The tripartite organization of these domains is evolutionarily conserved, suggesting an integrated and functional role for this overall protein structure. RING-finger and B-box motifs have been identified based on sequence homologies and are predicted to function as zinc-binding domains, but their precise functions have not been fully elucidated. There is evidence suggesting that the RING-finger, B-box and

coiled-coil domains mediate protein-protein interactions (Cao et al., 1997; Bartkiewicz et al., 1999).

Several RBCC proteins have been implicated in oncogenesis. The RBCC member PML becomes fused to the retinoic acid receptor alpha in acute promyelocytic leukemia (De The et al., 1991). Similarly, the RBCC proteins BRCA1, Cbl, Rfp, TIF1, and MDM2 have been demonstrated to be oncogenic when fused to other factors through chromosomal translocation events (Saurin et al., 1996). Other RBCC proteins have been implicated in signal transduction, E2-dependent ubiquitination, organellar biogenesis, chromosomal dynamics, viral pathogenesis, transcription, and developmental patterning (Saurin et al., 1996; Freemont, 2000).

Recently, a complex congenital human disease, Opitz G/BBB syndrome, was shown to result from mutations in the RBCC protein, Mid1 (Quaderi et al., 1997). Opitz G/BBB syndrome is characterized by abnormalities of midline structures, including hypertelorism, clefts of lip and palate, laryngotracheoesophageal defects, hypospadias, imperforate anus, and developmental delay. The Mid1 gene product is widely expressed during development and interacts with microtubules throughout the cell cycle (Cainarca et al., 1999). Overexpression of Mid1 leads to a stable population of microtubules resistant to depolymerization

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¹Abbreviations used in this paper: MAP, microtubule-associated protein; MEF2, myocyte enhancer factor 2; MHC, myosin heavy chain; MOI, multiplicity of infection; MURF, muscle-specific RING-finger protein; RBCC, RING-B-box-coiled-coil; SRF, serum response factor.

(Schweiger et al., 1999). Interestingly, mutations of Midl that are linked to Opitz G/BBB syndrome severely diminish the ability of Midl to interact with microtubules, suggesting that Midl-microtubule interaction and/or microtubule dynamics are involved in the processes required for normal development of the midline structures affected in Opitz G/BBB syndrome.

We describe a novel RBCC protein, MURF (muscle-specific RING finger) that shows significant homology to Midline proteins in the RING-finger and B-box regions, but is structurally distinct. MURF expression is restricted to cardiac and skeletal muscle throughout pre- and postnatal development. Like Midl, MURF binds and stabilizes microtubules against depolymerizing agents. Perturbation of MURF expression in C2 muscle cells inhibits myogenic differentiation. These properties of MURF suggest its involvement in microtubular stabilization in striated muscle cells, which previous studies have shown to be important for alignment of skeletal myoblasts during fusion, myofibrillar assembly, and contractile abnormalities in cardiac hypertrophy. The finding that MURF expression is required for execution of the muscle differentiation program also reveals a potential link between microtubule function and myogenesis.

Materials and Methods

Yeast Two-Hybrid Screen

Yeast two-hybrid screens of an adult mouse heart cDNA library were performed using full-length serum response factor (SRF) fused to the GAL4 DNA binding domain, as described previously (Spencer et al., 1999). Plasmids were isolated and transformed into electrocompetent XL1-Blue *E. coli*. Plasmids were prepared by alkaline lysis extraction and sequenced. Sequence analysis and comparison was performed using the NCBI web site Blast program.

We identified a cDNA encoding a novel muscle-specific RING-finger protein that interacted with GAL4-SRF. However, subsequent attempts to demonstrate interaction between SRF and MURF, or colocalization of the proteins in mammalian cells, were unsuccessful. Thus, we do not believe this interaction is biologically significant, but it may reflect an ability of SRF to interact with other RING-finger proteins, as several such proteins have been shown to regulate transcription (Hsieh et al., 1999). Nevertheless, because of its interesting structure and expression pattern (see Fig. 2), we continued to characterize this novel factor.

Mammalian Expression Plasmid Construction

Full-length MURF expression plasmids and COOH-terminal deletion mutants were constructed by PCR using Expand High Fidelity polymerase (Roche) and cloned in-frame into vector pcDNA3.1 myc/HIS (Invitrogen) or pECE-Flag. Native MURF was cloned into vector pcDNA1.1. NH₂-terminal deletion mutants were constructed by PCR, generating an initiator methionine at desired positions and cloned into the SacII and HindIII sites of full-length MURF in vector pcDNA3.1 myc/HIS. The SacII site is immediately downstream of the Kozak translation initiation sequence contained in the MURF cDNA. The Flag-tagged versions of MURF were subcloned into pcDNA3.1 for use in *in vitro* analyses.

Radioactive *In Situ* Hybridization

RNA probes corresponding to the sense and antisense strands of the MURF cDNA were prepared using T7 RNA polymerase (Roche). *In situ* hybridization was performed as previously described (Benjamin et al., 1997).

Cell Culture, Transfection, Alkaloid Treatment, and Immunofluorescence

Cos-1, HeLa, 10T1/2, 293, 3T3 and 293T cells were maintained in DME

supplemented with 10% heat-inactivated FBS. C2 cells were maintained in DME containing 20% heat-inactivated FBS. Cells were transfected with 1 μ g of various MURF expression plasmids using Fugene6 reagent as recommended by the manufacturer (Roche). 293T cells were transfected using the calcium phosphate coprecipitation technique (Spencer and Misra, 1996). Cells were treated for 2 h with 2 μ M nocodazole (Sigma-Aldrich) or 2 μ M cytochalasin D (Sigma-Aldrich) as indicated.

For immunofluorescence, cells were either extracted with 1% Triton X-100 in PBS for 1 min, washed twice in PBS, and fixed for 15 min in 3.7% formalin plus 0.25% glutaraldehyde (Sigma-Aldrich) in PBS or immediately washed with PBS and then fixed in 3.7% formalin solution. Cells were then washed three times in PBS and blocked in PBS containing 3% BSA and 0.1% NP40 for 30 min. All antibodies were incubated in fresh block solution for 60 min at ambient temperature. Myc-tagged versions of MURF were detected using antibody 9E10 (Santa Cruz) at a dilution of 1:250. Flag-tagged versions of MURF were detected using the M2 Flag antibody at a dilution of 1:500 (Kodak). Microtubules were detected using alpha-tubulin antibody at a dilution of 1:2,000 (Sigma-Aldrich). Intermediate filaments were visualized using vimentin antibody at a dilution of 1:500 (Sigma-Aldrich). Actin was visualized using rhodamine-conjugated phalloidin (Sigma). Myosin heavy chain was visualized using anti-myosin heavy chain antibody MY32 (Sigma-Aldrich) at a dilution of 1:250. Secondary antibodies were purchased from Vector labs and used at a dilution of 1:400 in fresh block solution. For cryo-sections of mouse quadriceps, MURF antibody UT82 was used at a dilution of 1:200 and alpha-tubulin antibody was used at a dilution of 1:500.

In Vitro Transcription/Translation and Immunoprecipitation

In vitro transcription/translation was performed as directed by the manufacturer (Promega). Plasmids used were native MURF in pcDNA1.1, COOH-terminally myc-tagged full-length MURF and various myc-tagged deletions of MURF. Immunoprecipitations were performed using ³⁵S-methionine-labeled *in vitro* translation products. In brief, ³⁵S-methionine-labeled products were incubated with agitation in 1 ml immunoprecipitation buffer (PBS, 0.5% Triton X-100, 0.25 mM zinc sulphate) at 4°C for 2 h. 2.5 μ l myc-antibody 9E10 was added and incubated an additional 60 min. 20 μ l protein A/G-plus beads (Santa Cruz) were added and incubated 60 min. Reactions were centrifuged at 2,500 rpm for 5 min at 4°C. Pellets were washed with rocking for 5 min in 1 ml immunoprecipitation buffer. Washing was repeated 4 times before resuspension of the pellet in 20 μ l SDS-sample buffer. Approximately 10 μ l was used in SDS-PAGE analysis. Gels were dried under vacuum and results were visualized by autoradiography.

Northern Blot Analysis, RNA Isolation and RT-PCR

Northern blot analysis was performed using a multiple tissue Northern blot from Clontech and ³²P-labeled probe made from the MURF full-length cDNA. Hybridization was performed for 1 h using rapid-hybe buffer (Amersham). C2 cells were induced to differentiate by the addition of DME supplemented with 2% horse serum. Cells were incubated for various lengths of time and total RNA isolated by TriZol extraction (Gibco). RT-PCR was performed as described in Rawls et al. (1998). Approximately 7 μ g total RNA was used in the slot blot analysis and hybridization conditions were as described above.

UT 82 Antibody Production

An NcoI-HindIII fragment spanning the coding region for amino acids 271–366 of MURF was fused in-frame into vector pGEX2T-polyG. BL21-DE3 cells containing the GST-fusion expression plasmid were grown to an optical density of 0.5 and induced with 1 mM IPTG for 3 h at 30°C. Cells were pelleted and washed in ice-cold PBS. Lysis was induced by sonication and protein was purified by GST-affinity chromatography using standard techniques. Rabbit immunization was conducted at Cocalico Biological.

Western Blot

Whole cell extracts from mouse tissues and C2 cells were prepared as described previously (Spencer et al., 1999). Approximately 25 μ g total protein was used in the analysis. MURF UT82 was used at a dilution of 1:4,000, anti-Glu-tubulin was used at a dilution of 1:2,000, anti-tubulin was used at a dilution of 1:10,000, and anti-myogenin (Santa Cruz) was used at a

dilution of 1:500. Mouse and rabbit HRP-conjugated secondary antibodies were used at a dilution of 1:5,000. Chemiluminescence was detected using Luminol Reagent (Santa Cruz).

Microtubule Sedimentation Assays

For endogenous MURF-microtubule cosedimentation experiments, 2-mo-old female mice were killed by standard protocols; hearts and quadriceps (100 mg each tissue) were removed, and placed in ice-cold PBS containing protease inhibitors (Roche) for 20 min. Tissue was finely minced and placed in 1 ml PCM buffer (0.1 M Pipes, pH 6.9, 2.5 mM CaCl₂, 1 mM MgSO₄, pH 6.9) plus protease inhibitors. This was promptly homogenized in a ground glass Dounce homogenizer with 25–30 strokes on ice. Nuclei and debris were removed by low speed centrifugation (3,000 rpm) for 5 min at 4°C. The supernatant was centrifuged at 100,000 *g* for 30 min at 4°C to remove cytoplasmic contaminants. The resulting supernatant was used in the microtubule sedimentation analysis. The supernatant was supplemented with 2 mM GTP and 5 mM EGTA and incubated at 37°C for 20 min. This was layered onto a 25% sucrose cushion in PEM buffer (0.1 M Pipes, 1 mM EGTA, 1 mM MgSO₄) plus 1 mM GTP and centrifuged at 20,000 *g* for 30 min at room temperature. The supernatant was removed and acetone precipitated. The pellet was resuspended in PEM buffer minus GTP and supplemented to 2 mM CaCl₂. This was placed on ice for 30 min. The second polymerization was induced by adding EGTA and GTP to 5 mM and 1 mM, respectively, and incubation at 37°C. Polymerization-depolymerization was repeated three times to avoid cytoplasmic contamination. The final polymerization was induced as described above and supplemented with 20 μM taxol (Sigma-Aldrich). The resulting microtubule pellet was resuspended in 50 μl PEM. Approximately 25 μg protein was used for Western analysis. For microtubule sedimentation assays using transfected cells, cells were harvested and treated as described in (Kaufmann et al., 1999).

Adenoviral Transduction

MURF cDNA was cloned in the antisense orientation into vector pAC-CMV.pLpA as a KpnI-XbaI fragment creating anti-MURF. The adenoviral expression plasmid encoding GFP (Ad-GFP) was constructed by cloning the GFP cDNA into vector pACCMV.pLpA. Replication-deficient adenovirus was created by cotransfecting plasmid JMI7 and anti-MURF into 293T cells. C2C12 cell infection was performed for 24 h in growth medium, followed by 5 washes in PBS and transfer to differentiation medium. Infection efficiency was monitored by fluorescence using Ad-GFP. Typically, 75–85% of the cells expressed GFP 48 h after infection.

Retroviral Transduction

The myogenin cDNA was cloned into vector MSCV/puro as an EcoRI fragment for virus production. Helper-virus free replication-defective retroviral supernatants were obtained by transient transfection of 293T cells with an ecotropic packaging construct. C2 cells were transduced in the presence of 8 μg polybrene for 3–4 h, then the medium was changed to fresh medium and cells allowed to recover overnight. Cells were selected for 3–6 d in the presence of 2 μg/ml puromycin. Expression of myogenin was confirmed by Western blot analysis.

Results

MURF Domain Structure and Similarity to Midline 1 and 2

The MURF cDNA encodes a 366-amino acid protein with a predicted molecular mass of 41 kD and pI of 4.82 (Fig. 1 A, data are available from GenBank/EMBL/DBJ under accession number AF294790; see Materials and Methods for method of identification of MURF). MURF contains several domains that identify it as a RBCC-type of RING-finger protein. A RING-finger of the C₃HC₄ type is located near the NH₂ terminus (amino acids 26–81), followed by another type of zinc-finger termed a B-box (amino acids 126–158; Fig. 1 B). In all other RBCC pro-

teins, the spacing between the RING-finger and B-box is also ~40 amino acids (Borden, 1998; Saurin et al., 1996). A predicted leucine-rich coiled-coil domain (amino acids 212–253) and an acidic region (amino acids 335–366) are located in the COOH-terminal portion of the protein.

Database searches with the amino acid sequence of MURF revealed highest homology to the Opitz-G/BBB syndrome protein Mid1 and the related factor Mid2 (Fig. 1, C and D) with greatest homology in the RING-finger and B-box domains. Interestingly, MURF does not contain a region corresponding to the first B-box of Mid1 and Mid2 nor the butyrophilin-like domain at the COOH termini of Mid2 and Mid2, suggesting functional differences between the proteins.

MURF Expression Is Restricted to Striated Muscle

The expression pattern of MURF was examined during mouse embryogenesis by *in situ* hybridization. At E8.5, MURF expression was observed only in the developing cardiac region and at E10.5 expression was restricted exclusively to the heart and the myotome of the somites which gives rise to skeletal muscle. This muscle-specific expression continued throughout prenatal development, with expression observed in the heart and skeletal muscle of the intercostals, diaphragm, limbs, face, and head (see Fig. 2 A).

In adult mice, Northern analysis showed a single MURF transcript of ~1.5 kb in cardiac and skeletal muscle (Fig. 2 B). Extended exposures (>6 d) revealed a very low level of expression in the lung and brain (not shown). Consistent with the restricted expression of MURF mRNA, Western blot analysis of protein from mouse heart, quadriceps, spleen and lung, using anti-MURF antibody, detected MURF protein only in heart and skeletal muscle (Fig. 2 C). The size of the protein, 41 kD, was in agreement with the size predicted from the open reading frame.

We also examined expression of MURF during differentiation of the C2 skeletal muscle cell line. As shown in Fig. 2 D, MURF transcripts, measured by semi-quantitative RT-PCR, were expressed in a pattern that mirrored that of MyoD. Like MyoD, MURF expression was detected in proliferating myoblasts before expression of myogenin and was upregulated during myoblast differentiation. MURF expression was also observed well before the expression of muscle differentiation genes such as skeletal α-actin, suggesting an early role for MURF in myogenesis. This is consistent with expression observed in the myotome and with functional studies described below.

MURF Is a Necessary Component of Myogenic Differentiation and Myotube Formation

Given the early expression of MURF in the developing myotome and increased expression of MURF during skeletal muscle differentiation *in vitro*, we investigated whether MURF might play a regulatory role during differentiation. To investigate this possibility, MURF expression was ablated by an adenovirus-delivered MURF antisense expression vector in C2 cells. Myoblasts were infected for 24 h and then switched to differentiation media for various lengths of time. As seen in Fig. 3 A, efficient expression of the MURF antisense transcript was detected 1 day after

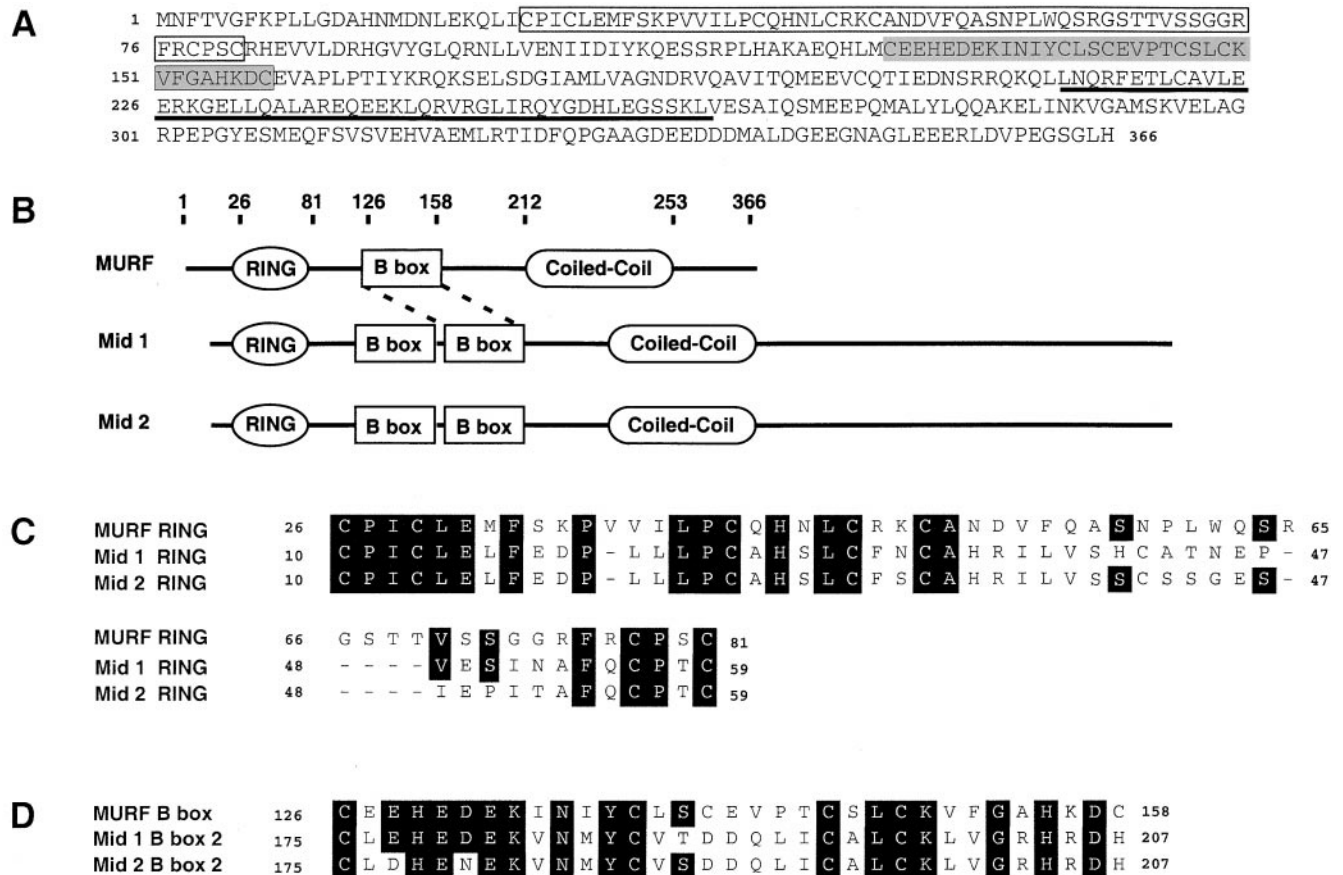


Figure 1. Amino acid sequence of MURF and structural similarity to Midline proteins. (A) Deduced amino acid sequence of MURF. The RING-finger domain is boxed, the B-box domain is shaded, and the coiled-coil domain is underlined. (B) Schematic diagrams of MURF and Midline proteins. (C and D) Amino acid homologies between the RING-finger and B-box domains, respectively, of MURF and Midline proteins.

adenovirus infection and was maintained for 2–4 d after infection, decreasing in expression with time. Western blot analysis showed that MURF protein expression was efficiently inhibited by antisense treatment for up to 3 d post-infection.

To determine if the absence of MURF protein had an effect on myotube formation, immunofluorescence was performed to detect the late muscle marker myosin heavy chain (MHC). The ablation of MURF expression severely diminished MHC expression (Fig. 3, compare B with C) and concomitant myotube formation. The stage at which differentiation was inhibited was also assessed by measuring muscle transcripts by RT-PCR. As seen in Fig. 3 D, compared with untreated or AdGFP-infected cells, MURF ablation inhibited the expression of the early myogenic regulatory factors, MyoD and myogenin. These data suggest that MURF is required for the initiation of myogenesis *in vitro*.

We next created stable C2 cells constitutively expressing myogenin to investigate whether constitutive expression of myogenin, an essential myogenic regulator, could bypass the requirement of MURF for initiation of myogenesis (see Fig. 3, E and F). Ablation of MURF expression in this background resulted in expression of MHC without myotube formation. Interestingly, while MHC was ex-

pressed, many cells showed collapsed MHC localization compared with uninfected myogenin overexpressing cells, suggesting that cytoskeletal integrity was compromised. These data, taken together, demonstrate that MURF has a role in myogenesis at two separate steps, initiation and myotube formation.

Endogenous MURF Cosediments with and Colocalizes with Microtubules from Striated Muscle

Given the amino acid homology between Mid1 and MURF and the ability of Mid1 to interact with microtubules (Cainarca et al., 1999; Schweiger et al., 1999), we investigated whether MURF was also a microtubule-binding protein. To determine if MURF associated with microtubules, microtubule sedimentation assays were performed using soluble extracts prepared from mouse skeletal muscle and heart. As seen in Fig. 4 A, MURF was contained in the microtubule pellet from these muscle extracts and was absent from the supernatant, demonstrating a physical association between MURF and microtubules *in vivo*. No contaminating actin filaments or intermediate filaments were detected in the microtubule pellets under these experimental conditions (not shown). Interestingly, the majority of MURF was contained in the initial high speed

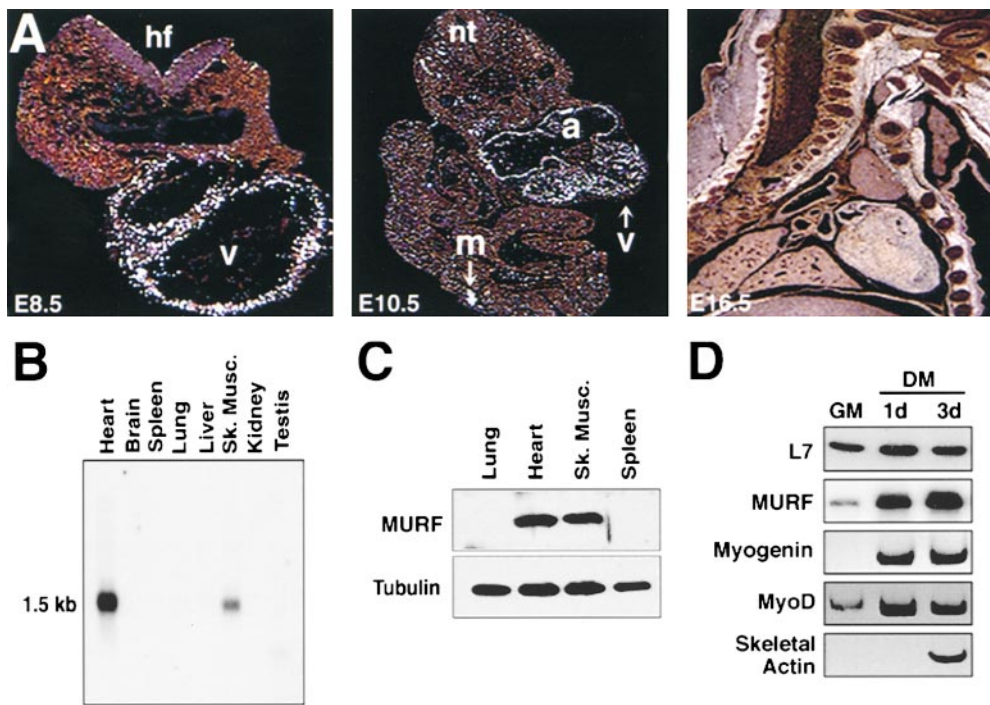


Figure 2. Muscle-specific expression of MURF RNA and protein. (A) Detection of MURF transcripts by in situ hybridization to mouse embryo sections. A transverse section through an E8.5 embryo shows expression exclusively in the cardiac forming region. A transverse section through an E10.5 embryo shows expression in the heart and myotomes. A sagittal section through an E16.5 embryo shows expression throughout skeletal muscle and heart. a, atria; m, myotome; hf, head folds; nt, neural tube; v, ventricle. (B) Northern analysis of poly(A)⁺ mRNA from adult mouse tissues. A 1.5-kb MURF transcript is detected only in heart and skeletal muscle. (C) Western blot analysis of MURF protein in extracts from adult mouse tissues. The 41-kD MURF

protein is detected only in heart and skeletal muscle. Blotting with anti-tubulin antibody confirmed equal quantities of protein in each lane. (D) RT-PCR analysis of RNA from C2 cells in growth medium (GM) or differentiation medium (DM) for 1 or 3 d, as indicated. MURF transcripts are detected at low levels in undifferentiated myoblasts in GM and are upregulated during differentiation. L7 is expressed constitutively and is a control for RNA loading.

pellet before microtubule polymerization, suggesting its association with cold-stable microtubules that sediment in the initial high speed centrifugation or with structures in addition to microtubules (see below).

In agreement with the apparent association of MURF with microtubules in skeletal muscle extracts (Fig. 4 A), immunostaining of cryosections of mouse quadriceps revealed a portion of MURF colocalizes with microtubules in vivo (Fig. 4, B–D). Consistent with MURF being present in the initial high speed pellet before microtubule polymerization, MURF was also associated with Z-lines in skeletal muscle. This raises the interesting possibility that MURF links microtubules to the Z-line in striated muscle by interacting with known or novel Z-line components (manuscript in preparation).

The Leucine-rich Coiled-Coil Domain Is Necessary and Sufficient for Interaction with Microtubules

Given that endogenous MURF cosedimented with microtubules from striated muscle, we next mapped the domain of MURF required for association with microtubules. 293T fibroblasts were transfected with plasmids expressing wild-type and deletion mutants of Myc-tagged MURF, simultaneously treated with nocodazole for 24 h to prevent microtubule association, and soluble extracts prepared. As seen in Fig. 5 C, full-length MURF cosedimented with microtubules in transfected cells. All MURF deletion mutants containing the leucine-rich coiled-coil domain also cosedimented with microtubules. Interestingly, mutant N212, which contains only the leucine-rich coiled-coil do-

main, was also partially contained in the supernatant after microtubule pelleting (Fig. 5 C, lane 5). Since mutant N167 was localized exclusively to the microtubule pellet, this suggests that although the leucine-rich coiled-coil domain is sufficient for association with microtubules, this domain also requires amino acids 168–211 for optimal interaction. Mutant C154, lacking the COOH-terminal leucine-rich coiled-coil domain, failed to pellet with microtubules and was contained exclusively in the supernatant. These data further demonstrate that MURF is capable of cosedimenting with microtubules, the leucine-rich coiled-coil domain is both necessary and sufficient for microtubule association, and maximal binding requires amino acids 168–211 under experimental conditions used.

The RING-Finger Domain Is Required for Continuous Interaction along the Entire Length of the Microtubule

To determine if deletions of MURF used in Fig. 5 changed the subcellular distribution of MURF, HeLa cells were transfected and immunofluorescence performed. As seen in Fig. 6 A, MURF was incorporated into an undulating reticular network in the cytoplasm, reminiscent of the subcellular distribution of Mid1 and Mid2 (Buchner et al., 1999; Cainarca et al., 1999; Schweiger et al., 1999). This network was extremely stable, remaining intact even after extraction with 1% Triton X-100 (data not shown). The resistance to extraction in high detergent concentrations suggested that MURF associated with a cytoskeletal component.

To determine if MURF colocalized with microtubules in

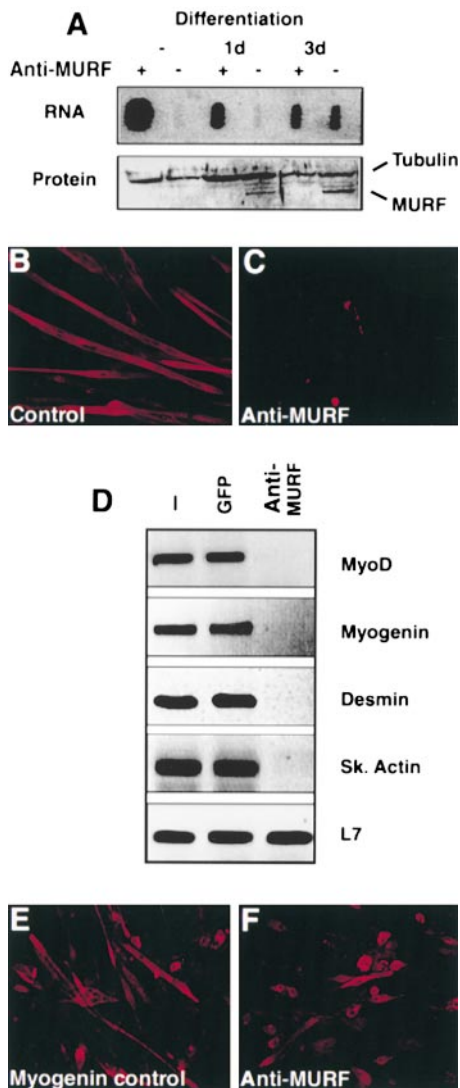


Figure 3. MURF expression is required for the initiation of myogenesis and myotube formation. (A) C2 myoblasts were infected at a multiplicity of infection (MOI) of 200 with an adenovirus expressing antisense RNA to MURF (Anti-MURF) for 24 h in growth medium and switched to differentiation medium. RNA and protein extracts were isolated at 0, 1, or 3 d of differentiation, and RNA slot blot and Western blot analyses were performed to detect MURF RNA and protein expression. MURF antisense transcripts are efficiently expressed immediately after infection and continue up to 3 d after infection. MURF protein expression is severely reduced in the presence of Anti-MURF for up to 3 d after infection. C2 myoblasts were infected with Ad-GFP as a control (B) or Anti-MURF (C) and induced to differentiate for 3 d. Cells were fixed and immunofluorescence performed using anti-myosin heavy chain antibodies to visualize myotube morphology. Inhibition of MURF expression blocks the accumulation of myosin heavy chain and myotube formation. (D) RT-PCR analysis of myogenic markers at day 3 of differentiation of mock-infected, Ad-GFP-infected, or Anti-MURF-infected C2 cells. Mock-infected and Ad-GFP-infected C2 cells express the myogenic markers MyoD, myogenin, desmin, and skeletal α -actin. Anti-MURF-expressing C2 cells fail to express these myogenic markers. L7 transcripts are expressed constitutively and serve as a control for RNA input. C2 cells engineered to overexpress myogenin were infected with Ad-GFP (E) or Anti-MURF (F) and immunofluorescence performed to detect skeletal myosin

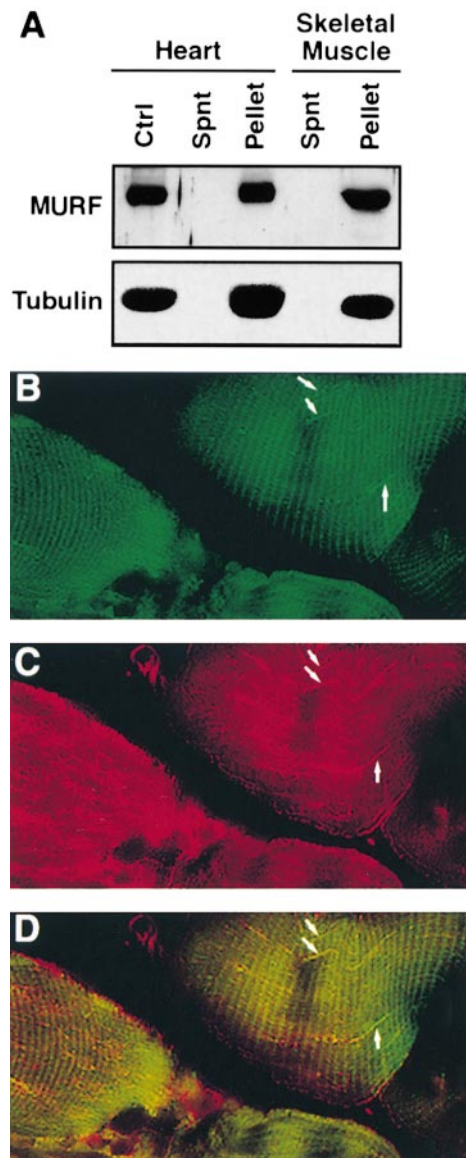


Figure 4. Association of MURF with microtubules demonstrated by microtubule sedimentation assay and immunolocalization of MURF in skeletal muscle. (A) Microtubule cosedimentation of endogenous MURF protein from striated muscle. Microtubules from soluble extracts from striated muscle were induced to polymerize as described in Materials and Methods. MURF was contained in the microtubule pellet from striated muscle. Ctrl lane was loaded with heart extract mixed with purified tubulin before polymerization. (B–D) Localization of MURF to microtubules in skeletal muscle by immunofluorescence with anti-MURF antibody UT82 raised against the COOH-terminal 95 amino acids of MURF, as described in Materials and Methods. (B) MURF staining alone. (C) Microtubules staining alone. (D) Overlay of B and C. The white arrows indicate regions of direct overlap of MURF staining and microtubule staining. MURF is localized to microtubules as well as Z-lines in skeletal muscle.

heavy chain expression as in B and C. C2 cells overexpressing myogenin differentiate normally and express myosin heavy chain. Blocking MURF expression in myogenin overexpressing cells results in a decrease in myotube formation but expression of MHC was unaffected.

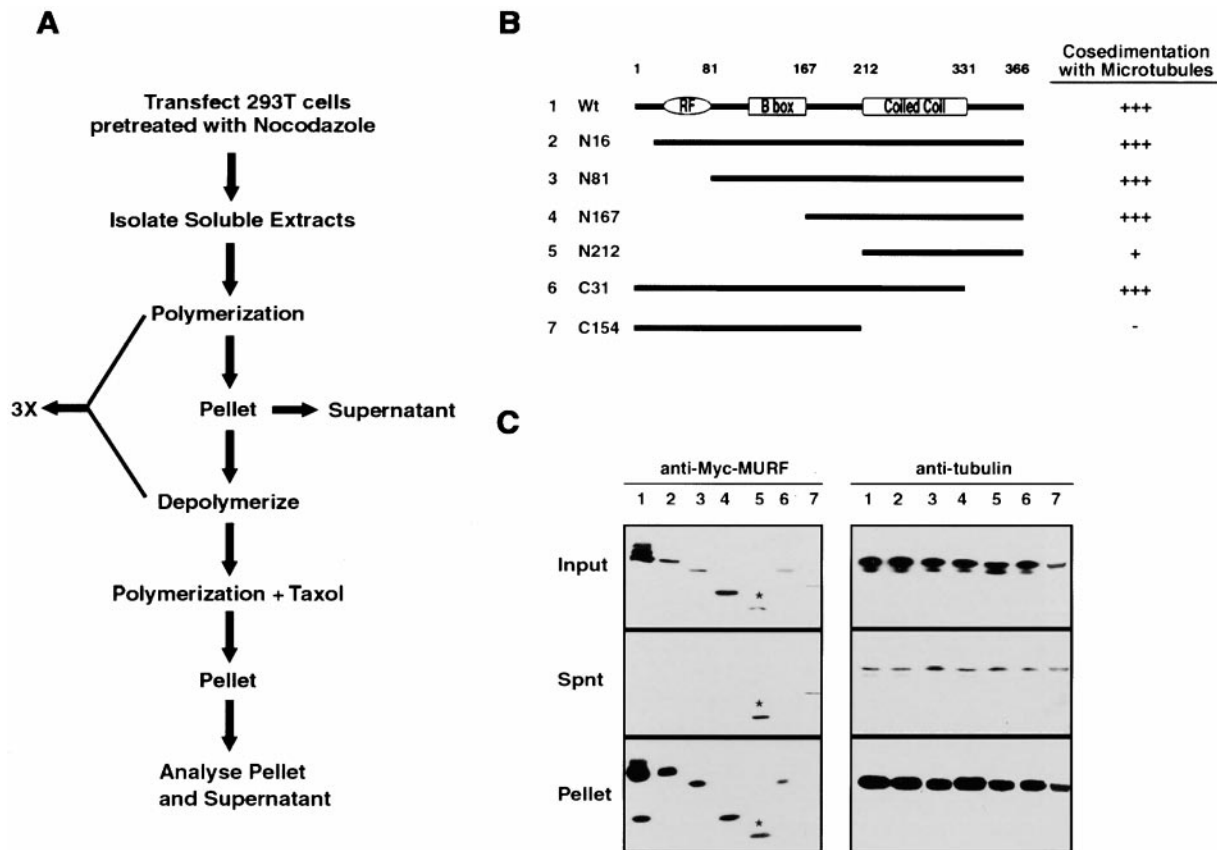
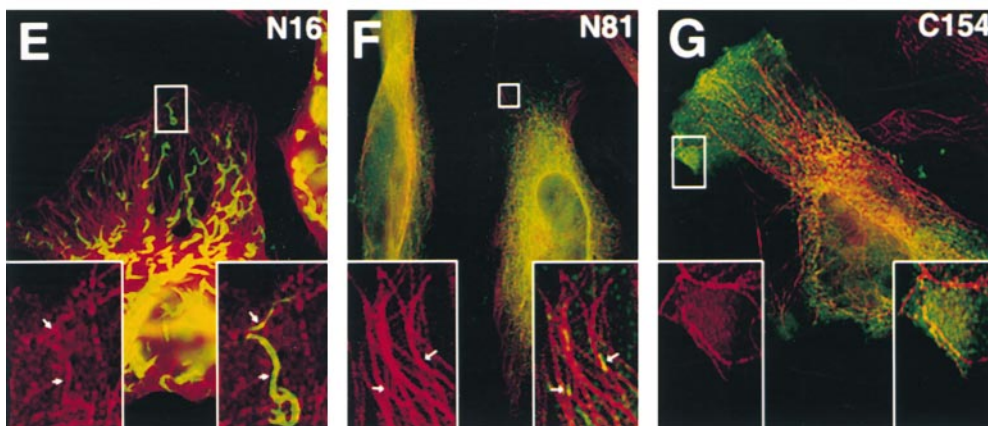
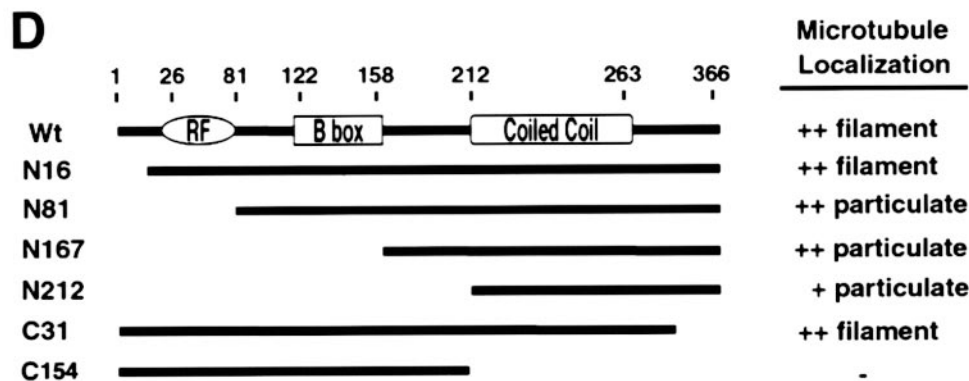
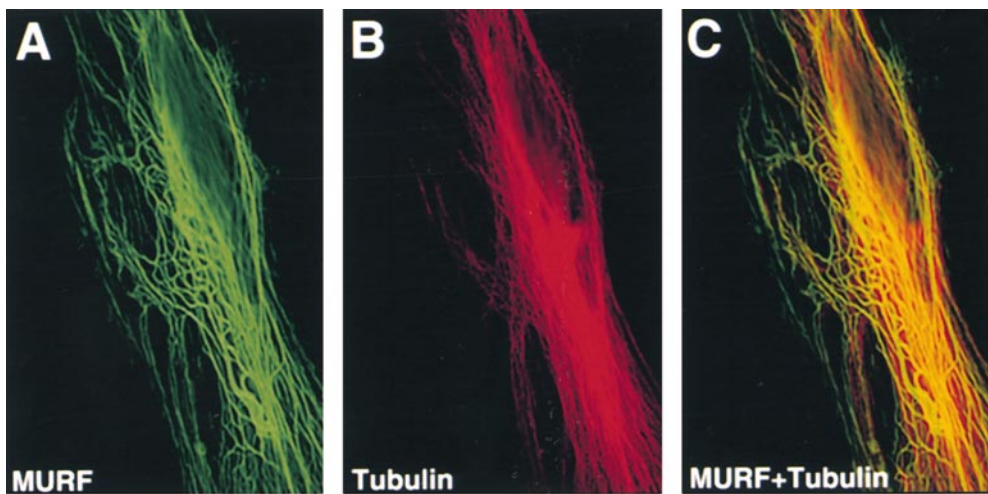


Figure 5. Mapping of domains of MURF required for association with tubulin by cosedimentation. (A) Schematic representation of the experimental protocol used in the *in vitro* microtubule cosedimentation assay. 293T cells treated with nocodazole were transfected with various myc-tagged versions of MURF and soluble extracts prepared. Purified tubulin was added and microtubules were polymerized in the presence of GTP and EGTA. Polymerized microtubules were pelleted and the resulting supernatant was acetone-precipitated. The microtubule pellet was subjected to depolymerization by calcium and cold treatment. Three cycles of polymerization-depolymerization were repeated before Western analysis. (B) Representation of MURF deletions used in the analysis and summary of domains required for microtubule binding. The leucine-rich coiled coil domain is necessary and sufficient for microtubule interaction (lane 5) but requires amino acids 168–211 for optimal binding (compare lanes 4 and 5). (C) Western blot analysis of MURF cosedimentation with microtubules. MURF mutants containing the leucine-rich coiled-coil domain cosediment with microtubules. An equivalent amount of MURF mutant N212 is contained in the supernatant and microtubule pellet (lane 5), indicating that amino acids 168–211 are required for optimal microtubule association. Mutant C154, lacking the leucine-rich coiled-coil domain, fails to cosediment with microtubules. The blot was reprobbed for α -tubulin to assess the extent of microtubule polymerization. Densitometric analysis revealed >90% of the tubulin input was contained in the microtubule pellet.

this assay, coimmunofluorescence was performed using antibodies specific to tubulin. Microtubules showed strong colocalization with MURF (Fig. 6, B and C), in agreement with the results of *in vivo* microtubule cosedimentation, *in vitro* microtubule binding assays, and *in vivo* coimmunofluorescence (see Figs. 4 and 5). There was no overlap of MURF localization with actin and only partial overlap with intermediate filaments at regions where microtubules and intermediate filaments were in close proximity (data not shown). Similar localization of endogenous MURF to microtubules was observed in skeletal muscle (Fig. 4, B–D).

As shown in Fig. 6, D and E, deletion of the 16 NH₂-terminal residues of MURF (mutant N16) did not disrupt colocalization with microtubules. However, the tubular structures formed with mutant N16 MURF were discontinuous. The significance of this redistribution is unclear. This discontinuous pattern is also reminiscent of the mi-

tochondrial distribution along microtubules. Staining of MURF-transfected cells with Mito-tracker dyes revealed partial overlap of MURF and mitochondria. However, subsequent purification of mitochondria from mouse hearts revealed no MURF protein associated with the mitochondrial fraction (data not shown). Deletion of amino acids 1–81 (mutant N81), removing the RING-finger domain, also did not abolish microtubule association, but prevented continuous association along the length of the microtubule (Fig. 6, D and F). Truncation of MURF to amino acid 167 (mutant N167), deleting the B-box domain, resulted in similar microtubule association without continuous binding as seen with full-length MURF protein (Fig. 6 D, and data not shown), indicating that the B-box is not involved in microtubule binding. Deletion of amino acids 1–212 (mutant N212), leaving only the leucine-rich coiled-coil domain and acidic COOH terminus, again prevented continuous binding along microtubules without af-

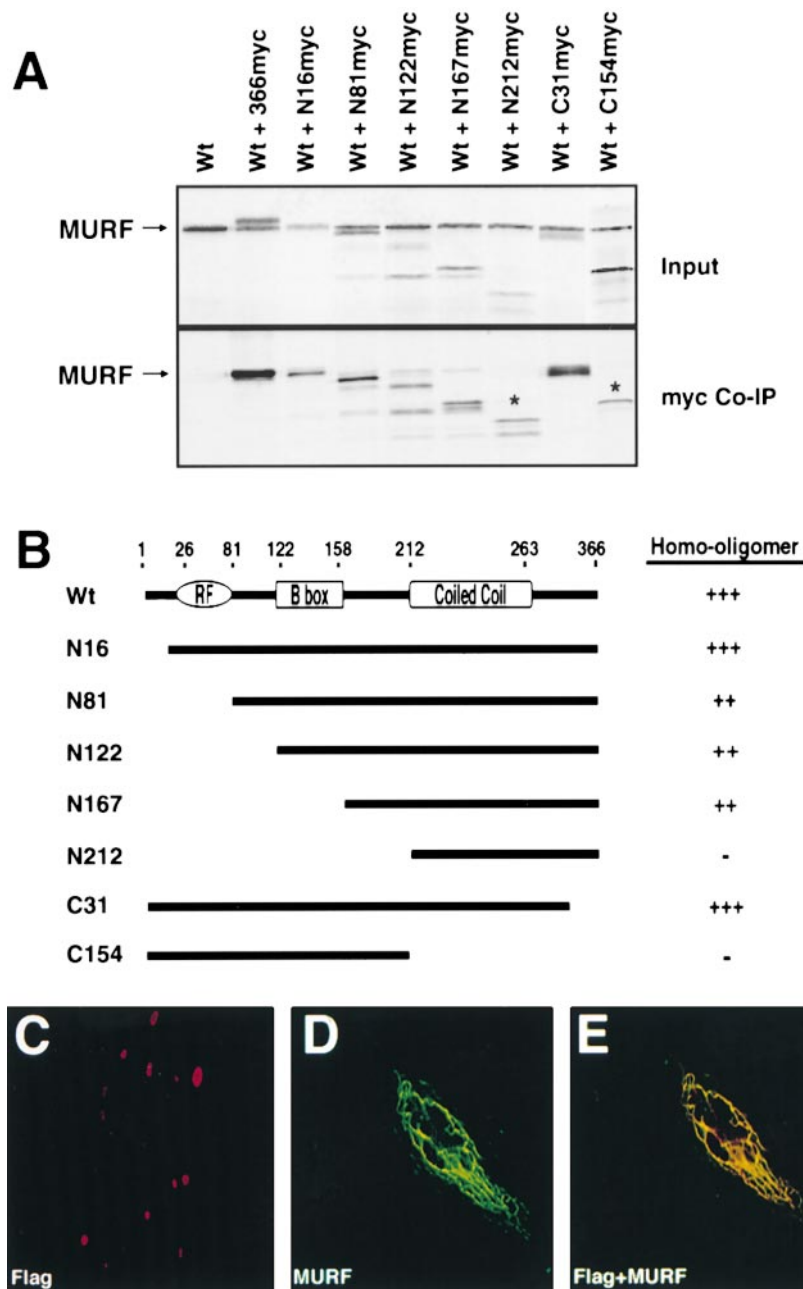


bule overlay. The arrows designate direct overlap of mutant N81 and microtubules. (G) Deletion of the leucine-rich coiled-coil (C154) prevents microtubule association. Mutant C154 is diffusely localized throughout the cytoplasm. The two larger white boxes in the corners of the panel are magnifications of the area of the cell outlined by the smaller white box. The left panel shows microtubules only, and the right panel shows MURF and microtubule overlay. Note MURF mutant C154 localization is diffuse and lacks organization. Identical results were obtained in multiple cell types.

fecting microtubule association (Fig. 6 D and see Fig. 4). Instead, as observed with mutants N81 and N167, particulate structures were formed that colocalized with microtubules. Detergent extraction of transfected cells before fixation revealed that significant proportions of mutants N81, N167, and N212 were associated with microtubules and not free aggregates of protein (not shown). Deletion of the COOH-terminal acidic region (mutant C31) did not affect

the association of MURF with microtubules nor continuous binding along microtubules (Fig. 6 D, and not shown). Truncation of the acidic and coiled-coil region of MURF (mutant C154) produced diffuse cytoplasmic staining that did not colocalize with microtubules (Fig. 6, D and G). Mutant C154 was readily detergent extractable from the cytoplasm before fixation, indicating it is not bound to microtubules nor any other cytoskeletal component (not

Figure 6. Mapping of domains of MURF required for microtubule localization by immunofluorescence. (A–C) HeLa cells were transfected with myc-tagged MURF and immunofluorescence performed. MURF (green) directly colocalizes with microtubules (red) as seen in C. Yellow color in C is the result of direct overlap of MURF and microtubule localization. (D) Summary of deletion mapping of microtubule binding domain of MURF. All mutants containing the leucine-rich coiled-coil domain interact with microtubules, whereas mutant C154 lacking the coiled-coil domain does not. The RING-finger is required for continuous binding along microtubules. (E) Deletion of the NH₂-terminal 16 amino acids (N16) does not inhibit microtubule interaction but alters the filaments to a discontinuous assembly. The two larger white boxes are magnifications of the area of the cell outlined by the smaller white box. The left panel shows microtubules only and the right panel shows MURF and microtubule overlay. Note the direct overlap of microtubules and MURF protein designated by arrows. (F) Deletion of the RING-finger domain (N81) does not inhibit microtubule binding, but prevents continuous binding along microtubules. The two larger white boxes are magnifications of the area of the cell outlined by the smaller white box. The left panel shows microtubules only, and the right panel shows MURF and microtubule overlay. Note MURF mutant C154 localization is diffuse and lacks organization. Identical results were obtained in multiple cell types.



shown). Deletion analyses were performed in Cos, 10T1/2, and 3T3 cells with identical results. Taken together, these data confirm that the leucine-rich coiled-coil region of MURF is necessary and sufficient for colocalization with microtubules, whereas the RING-finger is required for continuous binding along microtubules. These data also demonstrate that in cells MURF associates with microtubules with the greatest affinity if the RING-finger domain is intact.

MURF Homo-oligomerization Is Mediated by Amino Acids 168–211 and the Leucine-rich Coiled-Coil Domain

Coiled-coil domains often mediate protein-protein interactions (Li et al., 1994; Cao et al., 1997; Bartkiewicz et al.,

1999). To determine if the leucine-rich coiled-coil domain might mediate homo-oligomerization of MURF, we performed coimmunoprecipitation experiments using Myc-tagged MURF and native MURF translated in vitro. As summarized in Fig. 7, A and B, MURF was able to self-associate. Deletion of the RING-finger, and B-box (mutants N81, N122, and N167) did not affect homo-oligomerization, whereas deletion of amino acids 168–211 abolished homo-oligomerization (mutant N212). Interestingly, MURF lacking the leucine-rich coiled-coil domain but retaining amino acids 168–211 failed to homo-oligomerize. These data demonstrate that amino acids 168–211 and the leucine-rich coiled-coil domain comprise the homo-oligomerization domain of MURF. Of note, MURF containing the RING-finger domain was able to be pelleted at high speed in the absence of precipitation while

mutant N81, lacking the RING-finger, was not (not shown), suggesting that although the RING-domain is not required for homo-oligomerization, it may mediate the formation of large MURF-containing aggregates. This is consistent with the lack of continuous microtubule binding by mutants lacking the RING-finger domain.

While constructing epitope-tagged versions of MURF, we observed that the Flag-tag placed at the NH₂ terminus of MURF completely inhibited interaction with microtubules, resulting in formation of protein aggregates (see Fig. 7 C). Therefore, to determine if MURF was capable of associating with microtubules as a homo-oligomer, the NH₂-terminal Flag-tagged MURF and the COOH-terminal myc-tagged MURF were transfected separately or in combination into HeLa cells and immunofluorescence was performed. As seen in Fig. 7 C, the Flag-tagged MURF did not significantly colocalize with microtubules, instead forming large aggregates. In contrast, coexpression of Flag-MURF and the COOH-terminal myc-tagged version resulted in colocalization of both tagged version along the same filamentous structures (Fig. 7, C and D). This demonstrates that MURF is capable of associating with microtubules as a homo-oligomer. These data, taken together with Fig. 5, demonstrate that MURF associates with microtubules most efficiently as a homo-oligomer.

MURF Stabilizes Microtubules

Recently, Midl was shown to associate with and stabilize microtubules (Cainarca et al., 1999; Schweiger et al., 1999). Given the homology of MURF with Midl and the ability of endogenous MURF to colocalize and sediment with microtubules, we determined if MURF was able to

stabilize microtubules in cultured cells. Cos cells were transfected with MURF expression plasmid, cultured for 2 h in the presence of 2 μ M nocodazole, detergent extracted, and MURF expression was examined by immunofluorescence. As shown in Fig. 8, A–C, MURF prevented depolymerization of microtubules in the presence of nocodazole. In untransfected cells lacking MURF, microtubules were completely depolymerized as indicated by the absence of microtubule staining. Similar results were obtained using cold incubation or calcium treatment of detergent-extracted cells to destabilize microtubules (not shown). Of note, the microtubules bound by MURF appear much thicker relative to non-MURF bound microtubules, suggesting MURF bundles microtubules. This was only observed in Cos cells and not HeLa, 10T1/2, nor NIH3T3 fibroblasts. Therefore, we believe the bundling observed is cell line-dependent and not a general property of MURF.

To identify the domain of MURF required for microtubule stabilization, the previously described deletion mutants of MURF were examined for their ability to protect microtubules from depolymerization in transfected Cos cells. MURF mutants lacking the NH₂-terminal 16 amino acids or the COOH-terminal 31 amino acids (mutants N16 and C31) were able to protect microtubules from destabilization to a degree comparable to that of full-length MURF (Fig. 8 D). Interestingly, deletion mutants of MURF lacking the RING-finger (N81) did not stabilize microtubules. This demonstrates that the microtubule stabilizing effects of MURF are dependent on the ability to continuously bind along microtubules. As expected, deletion of the leucine-rich coiled-coil domain (C154), required for microtubule association, did not stabilize microtubules.

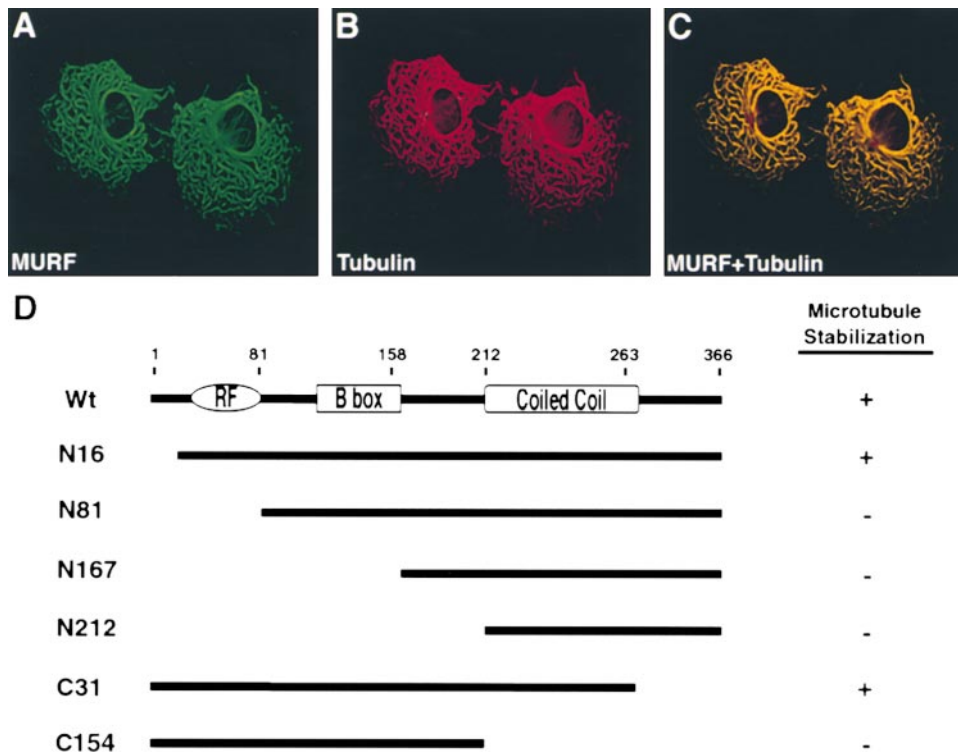


Figure 8. Mapping domains of MURF required for microtubule stabilization. (A–C) Expression of MURF stabilizes microtubules. Cos cells were transfected with a MURF expression plasmid, treated with 2 μ M nocodazole for 2 h and immunostained for MURF and tubulin. As seen in C, MURF is able to protect microtubules from alkaloid-induced destruction. Nontransfected cells did not contain intact microtubules as detected by the absence of tubulin staining. (D) Summary of the domains of MURF required for microtubule stabilization. Microtubule interaction and filament formation are required for microtubule stabilization. Deletion of the RING-finger domain (mutant N81), abrogating filament formation, or deletion of the leucine-rich coiled-coil domain (mutant C154), preventing microtubule association, ablates the microtubule stabilization function of MURF.

MURF Association with Microtubules Leads to the Formation of Glutamic Acid-modified Tubulin

Microtubules exist in several modified forms. The majority of the tubulin contained within microtubules is tyrosinated at its COOH terminus. Tubulin that has been detyrosinated can possess a glutamic acid residue at its COOH terminus (Glu-tubulin) or this residue can be removed (Δ_2 -tubulin). Microtubules composed of modified tubulin, such as Glu-tubulin and Δ_2 -tubulin, have been demonstrated to be more stable than unmodified or tyrosinated tubulin (Schulze et al., 1987; Webster et al., 1987; Webster, 1997). The more stable the microtubule, the longer it exists without depolymerization, and the greater the accumulation of modified tubulin within the microtubule. Therefore, the degree of modification is indicative of the age of the microtubule. As another measure of MURF's ability to stabilize microtubules, cells were transfected with MURF or various deletion mutants of MURF

and immunostained with antibodies specific to the glutamic acid-modified form of tubulin (Glu-tubulin). As seen in Fig. 9 C, only cells possessing the tubular network of MURF-bound microtubules contain Glu-tubulin, suggesting these microtubules are indeed stable and have existed for a significant period without depolymerization. Deletion of the RING-finger domain did not lead to Glu-tubulin accumulation (Fig. 9 D, and not shown.) Microtubule binding is required for Glu-tubulin accumulation as deletion C154 did not increase Glu-tubulin formation (Fig. 9 D, and not shown). These data are consistent with the results of Fig. 8 demonstrating that MURF is able to stabilize microtubules and continuous binding along microtubules is required for this process to occur. Identical results were obtained using a Δ_2 -tubulin antibody (not shown).

To determine if MURF expression parallels the formation of Glu-modified microtubules during skeletal muscle differentiation, C2 cells were isolated at various stages of

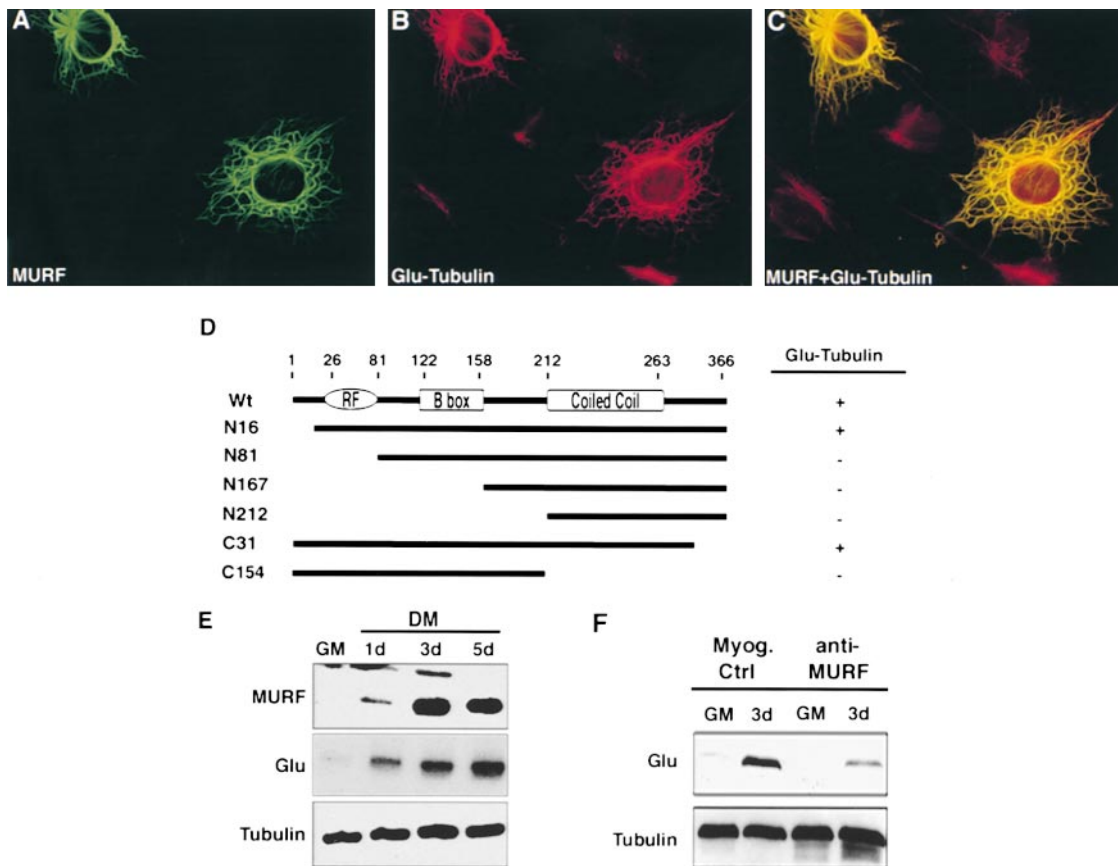


Figure 9. Stabilization of microtubules by MURF in vivo. (A–C) MURF expression enhances Glu-tubulin formation. Cos cells were transfected with a MURF expression plasmid and immunostained for MURF and Glu-tubulin to detect stable microtubules 24 h after transfection. A is MURF localization. B is the accumulation of Glu-tubulin. C is an overlay of A and B. (D) Summary of the domains of MURF required for stabilization of microtubules as detected by the accumulation of Glu-tubulin. The RING-finger and leucine-rich coiled-coil are required to observe Glu-tubulin accumulation. (E) MURF expression parallels the appearance of Glu-tubulin during skeletal muscle formation in vitro. C2 cells were grown in growth medium (GM), induced to differentiate for 1, 3, or 5 d and Western blot analysis performed to detect MURF expression and Glu-tubulin. MURF expression and Glu-tubulin accumulation occur in concert. Blotting with an anti-tubulin antibody confirmed equal quantities of protein in each lane. (F) Ablation of MURF expression in myogenin overexpressing C2 cells results in decreased Glu-tubulin accumulation. MURF expression was inhibited in myogenin overexpressing C2 cells by anti-MURF as described in Materials and Methods. Extracts were isolated from mock-infected and anti-MURF-infected cells at 0 (GM) and 3 d of differentiation and Glu-tubulin levels were detected by Western blot. Total tubulin levels were used as a loading control. Glu-tubulin levels were normalized to total tubulin by densitometry.

differentiation and Glu-tubulin was analyzed by Western blot analysis. As seen in Fig. 9 E, the appearance of Glu-tubulin mirrors the expression of MURF during muscle differentiation. These data raise the possibility that MURF/microtubule interaction may participate in the mechanism regulating microtubule equilibrium during the formation of striated muscle.

Given the parallel expression of MURF with the appearance of Glu-tubulin in skeletal muscle differentiation and the formation of stable microtubules in MURF-expressing cells, we investigated if the ablation of MURF expression affected the formation of stable microtubules during myogenic differentiation in myogenin overexpressing muscle cells. Western blot analysis was performed to detect the accumulation of Glu-tubulin as a marker of microtubule stability at 0 and 3 d of differentiation. As seen in Fig. 9 E, in mock-infected cells Glu-tubulin accumulation is robust after three days of differentiation. In contrast, in anti-MURF-infected cells capable of expressing myogenic markers but unable to form myotubes, Glu-tubulin formation was significantly reduced. Normalization of the levels of Glu-tubulin to total tubulin concentration revealed an ~4–5-fold decrease. These data demonstrate that MURF expression during differentiation is an integral component in the establishment of stable microtubules required for myotube formation.

Discussion

The results of this study identify MURF as a novel muscle-specific RING-finger protein that stabilizes microtubules and is essential for the initiation of myogenesis and myotube formation. The selective expression of MURF in differentiated striated muscle suggests its involvement in establishment of the stable microtubular network known from previous studies to influence myofibrillar assembly, contractility, and morphology of muscle cells (Antin et al., 1981; Toyama et al., 1982).

MURF and Midline Proteins

MURF contains several distinct domains that contribute to the stabilization of microtubules (Fig. 10). Microtubule binding and homo-oligomerization are mediated by overlapping functional domains. The leucine-rich coiled-coil is necessary and sufficient for microtubule interaction. However, amino acids 168–211 are required for optimal association. Homo-oligomerization also is mediated by the leucine-rich coiled-coil and amino acids 168–211. In contrast to microtubule binding, the leucine-rich coiled-coil is not sufficient for homo-oligomerization *in vitro*. Continuous binding along microtubules *in vivo* requires the RING-finger domain of MURF.

Although MURF and Midline RING-finger proteins share homology and the ability to associate with microtubules, their overall structures are distinct, suggesting that their functions are not identical. MURF contains one B-box, whereas Midline proteins contain two, and Midline proteins contain a COOH-terminal Ret-finger protein (RFP) or butyrophilin-like domain not contained in MURF. The expression patterns of MURF and Midline proteins are also different. Whereas MURF expression is restricted to

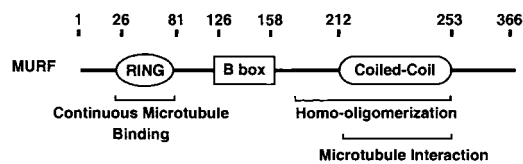


Figure 10. Functional domain structure of MURF. MURF is composed of distinct functional domains. The leucine-rich coiled-coil domain mediates microtubule association. Amino acids 168–211 cooperate with the leucine-rich coiled-coil domain in mediating homo-oligomerization. The RING-finger domain is required for continuous binding along and stabilization of microtubules.

striated muscle pre- and postnatally, Mid1 and Mid2 are more ubiquitous (Quaderi et al., 1997; Buchner et al., 1999). Embryonically, Mid1 is expressed in all developing tissues except the heart and Mid2 is highly expressed in the heart as well as elsewhere in the embryo.

Microtubules, MAPs, and Disease

The equilibrium of microtubules between polymerized and depolymerized states influences a variety of cellular processes, including morphological changes, migration, and proliferation. The microtubule-binding properties of MURF are similar in many respects to those of other microtubule-associated proteins, such as MAPs, Midline family members, Doublecortin, Lis1, and Tau, which bind and stabilize microtubules (Kosik, 1990; Takemura et al., 1992; Kaech et al., 1996; Sapir et al., 1997, 1999; Nguyen et al., 1997, 1998; Buchner et al., 1999; Cainarca et al., 1999; Gleeson et al., 1999; Hsieh et al., 1999; Koulakoff et al., 1999; Schweiger et al., 1999). However, MURF lacks the type of microtubule association domain commonly contained in MAPs but, instead, associates with microtubules through its leucine-rich coiled-coil domain. Other microtubule-binding proteins that lack the classic microtubule association domain found in MAPs have also been identified, including Mid1, Mid2, Doublecortin and Lis1. With the exception of Mid1 and Mid2, most nonclassical MAPs do not share significant homology with MURF, suggesting multiple mechanisms for microtubule interaction that may regulate microtubule dynamics in different tissues.

Several human diseases have been linked to mutations in genes encoding microtubule-binding proteins, demonstrating the importance of the microtubule network for normal patterning and development. For example, Mid1, Doublecortin, Lis1, and Tau, all microtubule-associated factors capable of stabilizing microtubules, have been implicated in the progression of disease (Kosik, 1990; Lo Nigro et al., 1997; Quaderi et al., 1997; Gleeson et al., 1999; Koulakoff et al., 1999; Tolnay and Probst, 1999). Mutant mice lacking the microtubule-binding proteins Kif3A and Kif3B of the kinesin superfamily, have also been shown to display situs inversus of the heart and other organs (Marszalek et al., 1999; Takeda et al., 1999). A similar phenotype is observed in mice harboring a deleted ATP binding domain of left-right dynein (Supp et al., 1999). The possibility that cardiomyopathies or muscular dystrophies may be linked to MURF expression remains an interesting prospect for the future.

The Cytoskeleton in Muscle

Microtubules have been shown to play important roles in regulating muscle cell morphology, sarcomere assembly, and function. In skeletal muscle cells, the microtubule network is reorganized to form stable microtubules during differentiation (Gundersen et al., 1989). Consistent with this requirement for stable microtubules, ablation of MURF expression disrupts skeletal muscle differentiation in vitro with a significant decrease in Glu-tubulin formation. Integrity of the microtubular array is essential for proper alignment of myoblasts during fusion and for correct formation of myofibrils (Antin et al., 1981; Toyama et al., 1982). However, given that MURF is expressed in cardiac muscle cells that do not undergo fusion events, the role of MURF likely extends beyond fusion alone.

By what mechanism might MURF affect myogenesis? It is well established that myoblast fusion requires precise cell-cell interactions, which are influenced by cell morphology. Thus, perturbation of MURF expression might distort myoblast morphology with consequent disruption of appropriate intercellular contacts. Alternatively, or in addition, aberrant cell shape or cytoskeletal architecture might perturb intracellular signaling systems that ultimately regulate muscle gene transcription. In this regard, activation of gene expression by SRF, a regulator of myogenesis, is linked to changes in actin dynamics and cytoskeletal reorganization (Sotiropoulos et al., 1999). In addition, microtubule disruption agents affect the activity of mitogen-activated protein (MAP) kinase family members, including p38 kinase, a known regulator of myocyte enhancer factor 2 (MEF2) transcriptional activity and myogenesis (Zetser et al., 1999; Stone and Chambers, 2000).

Consistent with the conclusion that MURF is an integral component of the mechanism that regulates microtubule stability in muscle cells, both skeletal and cardiac muscle have been demonstrated to possess a population of microtubules that is stable to depolymerization and cold shock (Webster, 1997). Moreover, expression of MURF mirrors the accumulation of stable glutamic acid-modified tubulin during myoblast differentiation in vitro and the ablation of MURF results in decrease in Glu-tubulin accumulation. A muscle-specific isoform of MAP4 is also expressed upon differentiation in a pattern very similar to that of MURF (Mangan and Olmsted, 1996). However, inhibition of MAP4 expression during differentiation does not block myotube formation but causes the accumulation of multinucleated myotubes with a rounded morphology, likely due to microtubule defects. In the MURF-ablated cells, there still is significant Glu-tubulin accumulation, although decreased. MAP4 may participate in the formation of Glu-tubulin observed in the MURF-ablated cells. The relationship between MAP4 and MURF is unclear.

Microtubule stabilization and increased microtubule density have also been proposed as a mechanism for contractile dysfunction in cardiac hypertrophy (Sato et al., 1997). There is evidence that stabilization of the microtubule array in hypertrophic cardiomyocytes is mediated by a microtubule-associated protein, that is as yet unidentified (Sato et al., 1997). MAP4, which can stabilize microtubules in vitro (Kaech et al., 1996; Nguyen et al., 1997; Nguyen et al., 1998), is induced during pressure overload-

induced hypertrophy (Sato et al., 1997), making it a plausible candidate for such a factor. Interestingly, inhibiting expression of MAP4 in fibroblasts does not adversely affect microtubule dynamics, suggesting a specialized role for MAP4 in a muscle environment or functional redundancy between members of the MAP family (Wang et al., 1996). The striated muscle-restricted expression pattern and microtubule stabilizing effects of MURF make it an interesting candidate for a factor contributing to contractile dysfunction in cardiac hypertrophy.

Microtubule depolymerization is also required for mitosis. Thus, the ability of MURF to prevent microtubule depolymerization is particularly intriguing in that striated muscle cells are irreversibly postmitotic. In this regard, MURF transfected cells are unable to divide (unpublished results). Whether MURF plays a role in preventing cell cycle reentry of differentiated cardiac and skeletal muscle cells is an interesting question for the future.

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