Identification of Muscle Specific Ring Finger Proteins as Potential Regulators of the Titin Kinase Domain

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The giant myofibrillar protein titin contains within its C-terminal region a serine-threonine kinase of unknown function. We have identified a novel muscle specific RING finger protein, referred to as MURF-1, that binds in vitro to the titin repeats A168/A169 adjacent to the titin kinase domain. In myofibrils, MURF-1 is present within the periphery of the M-line lattice in close proximity to titin’s catalytic kinase domain, within the Z-line lattice, and also in soluble form within the cytoplasm. Yeast two-hybrid screens with MURF-1 as a bait identified two other highly homologous MURF proteins, MURF-2 and MURF-3. MURF-1,2,3 proteins are encoded by distinct genes, share highly conserved N-terminal RING domains and in vitro form dimers/heterodimers by shared coiled-coil motifs. Of the MURF family, only MURF-1 interacts with titin repeats A168/A169, whereas MURF-3 has been reported to affect microtubule stability. Association of MURF-1 with M-line titin may potentially modulate titin’s kinase activity similar to other known kinase-associated proteins, whereas differential expression and heterodimerization of MURF1, 2 and 3 may link together titin kinase and microtubule-dependent signal pathways in striated muscles.

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Introduction

Titin is a large, 3000-3700 kDa myofibrillar protein consisting of a single 27,000-33,000 residue long polypeptide chain that spans the entire half-sarcomere. Titin’s N-terminal ~80 kDa region is integrated in the Z-line lattice of the striated muscle sarcomere, and its C-terminal ~200 kDa region is located in the M-line lattice (for reviews, see Trinick & Tskhovrebova1 and Gregorio et al.2). In addition to its structural functions, titin also confers elasticity to myofibrils by virtue of its elastic spring elements located in the I-band of the sarcomere (for a review, see Link & Granzier3). It is
interesting that titin also contains phosphorylation motifs, calpain-binding sites, and a serine/threonine-kinase domain, suggesting that titin may play a role in myofibrillar signal transduction.4-7

In particular, the presence of the catalytic serine/threonine kinase domain of titin, located within its C-terminal region in the M-line peripheral region of the sarcomere,8 has prompted numerous speculations regarding titin-based myofibrillar signal transduction pathways. For example, the homology of titin’s kinase domain to myosin-light chain kinase, and also the twitching phenotype of unc-22 mutants, a titin-homologous gene from Caenorhabditis elegans,9 raises the possibility that titin/twitchin kinase activities may regulate muscle contraction by phosphorylating myosin/myosin light chains. So far, phosphorylation of myosin/myosin light chains by vertebrate titin has not been demonstrated. It has also been suggested that altering levels of S100/Ca2+ complex dynamically controls twitchin/titin kinase activities, because vertebrate muscle S100 extracts were found to stimulate C. elegans twitchin kinase activity in vitro.10 It should be noted, however, that the complete C. elegans genome sequence does not contain S100 like genes.11 Recently, it was suggested that the physiological substrate of the titin kinase domain is the 19 kDa myofibrillar protein telethonin12 (also called T-cap),13 and that telethonin and its phosphorylation is critical for myofibrillogenesis.14 However, more recently, muscular dystrophy patients have been identified who are telethonin-null,15 yet their myofibrils are structurally normal. Therefore, the role of titin’s kinase domain remains elusive.

The kinase domain in the vertebrate titin polypeptide is flanked by Ig/FN3 domains with sequences and domain organizations that are conserved in titin, the titin-related invertebrate proteins projectin and twitchin, and in the smooth and non-muscle myosin light chain kinases.4,9,16,17 In titin, this region corresponds to the segment A168-A169-A170-kinase domain-M1-M2-M3 (A168, A169, M1, M2 and M3 all being Ig domains, whereas A170 is an FN3 domain; see Figure 1). Here, therefore, we searched for myofibrillar proteins that interact with baits containing titin’s A168/kinase/M3 region by using a yeast two-hybrid approach. This has led to the identification of the novel RING finger protein, MURF-1, that binds N-terminally of the titin kinase domain to the repeats A168/A169. MURF-1 therefore associates with the periphery of the M-line lattice and may be involved in the regulation of the titin kinase domain. Furthermore, since MURF-1 in turn forms heterodimers with two closely related MURFs, one of which has recently been shown to be involved in regulation of microtubule dynamics,18 the MURF family may link titin filament-based and microtubule-based signal transduction pathways.

![Figure 1](attachment:image.png)

**Figure 1.** Identification of a novel titin binding RING finger protein (MURF-1). (a) A titin construct spanning from the region A167 to M2 including the kinase domain was used as a bait for two hybrid screens. This identified MURF-1 as a specific binding partner. (b) The titin deletion constructs Δ1 to Δ8 showed that the titin repeats A168/A170 are required and sufficient for MURF-1 binding. (c) A central 144-residue segment of MURF-1 is required for binding to A168/A170 titin; MURF-1 binding to titin does not require the N-terminal RING finger or the C-terminal acidic domain. (d) In GST pulldown assays, the titin repeats A168/A169 were sufficient for interaction with MURF-1. Lane 1, size marker; lane 2, in vitro translated MURF-1; lane 3, GST pulldown assay using immobilized A168/A169; lane 4, control with immobilized GST; lane 5, control with immobilized titin N2A-construct.
Results

Specific interaction of MURF-1 and titin kinase flanking modules A168/A169

In order to identify sarcomeric proteins that may bind to the titin kinase region, yeast two-hybrid screens were performed with baits corresponding to titin A167 to the M2 region (Figure 1(a)). A total of 22 interacting clones were identified out of $1.6 \times 10^6$ screened clones. Four of the 22 clones could be confirmed by beta-galactosidase assays, and two of these four were shown by sequencing to encode the novel RING finger protein MURF-1 (for recent identification of a MURF, see Spencer et al.\textsuperscript{18} for a review on RING finger proteins, see Jackson et al.\textsuperscript{19}). The characterization of the two other interacting clones, i.e. whether they correspond to truly interacting clones or are false positives, is currently under study. To determine more precisely the MURF-1 binding site on titin, titin bait deletion constructs\textsuperscript{1} were tested. This showed that A168/A169 was necessary and sufficient for the interaction with MURF-1 (Figure 1(a) and (b)). Next, we determined the full-length cDNA sequence of MURF-1 by screening human heart and skeletal muscle cDNA libraries (see Materials and Methods). Its full length cDNA is 1.9 kb in length and encodes a 38 kDa RING finger protein (see Figures 2 and 3). Yeast two-hybrid interaction experiments using deletion constructs of MURF-1 with A168/A169 preys indicated that a central 144-residue segment of MURF-1 was required for its interaction with titin A168/A169 (Figure 1(c)).

Finally, we studied the interaction of the titin A168/A169 domains with MURF-1 by GST-pulldown assays. This \textit{in vitro} assay confirmed that A168/A169 titin sequences interacted with MURF-1 (Figure 1(d)).

MURFs, a gene family encoding muscle specific RING finger proteins

To identify proteins in addition to titin that may interact with MURF-1, we constructed a bait plasmid encoding 1.02 kb MURF-1 cDNA, containing the complete open reading frame of MURF-1 (Figure 2). When screening adult skeletal muscle cDNA libraries (9.2 $\times 10^5$ clones), two MURF-1 prey clones were identified, suggesting that MURF-1 may indeed homo-dimerize as indicated by its coiled-coil potential (Figure 2(a)). \textit{In vitro} GST-pulldown assays using $^{35}$S-labelled MURF-1 confirmed that MURF-1 indeed interacts with itself, presumably by forming homo-dimers (Figure 2(b)). By screening heart cDNA libraries with MURF-1 baits (2.8 $\times 10^6$ clones), we identified 11 positive clones. Among them, three clones were derived from the same gene and encoded a novel RING finger protein. This novel RING finger protein is 62% homologous to MURF-1 (Figure 3(a)), and is referred to here as MURF-2. Furthermore, we constructed a bait plasmid using full-length MURF-2 cDNA, and screened human skeletal muscle, brain, and mouse embryo cDNA libraries (5.1 $\times 10^5$,

Figure 2. Identification of MURF-1 binding proteins. (a) Two-hybrid screens with MURF-1 baits identified MURF-1 prey clones, suggesting formation of homo-dimers by the coiled-coil motifs. MURF-1 baits also identified two other homologous proteins, MURF-2 and MURF-3, which do not interact with A168-A170 titin. All three MURFs interacted with each other in the two-hybrid system, suggesting formation of heterodimers by the shared coiled-coil motifs. For MURF-2 and MURF-3, some of the sequenced cDNA clones had insertions with open reading frames, suggesting alternatively spliced isoforms (shown as MURF-2 and MURF-3, respectively). (b) Interaction of MURF-1 with itself was confirmed by GST-pulldown assays. Lane 1, size marker; lane 2, \textit{in vitro} translated MURF-1; lane 3, GST pull-down assay using immobilized MURF-1; lane 4, control with immobilized GST. (c) Interaction of MURF-1 and MURF-3 in GST-pulldown assays. Lane 1, GST pulldown assay using labeled MURF-1 and GST-MURF-3 fusion peptide. The binding of MURF-1 to glutathione-Sepharose 4b beads indicates interaction of MURF-1 with MURF-3, presumably by heterodimer formation. Lane 2, control with labelled MURF-1 and GST peptide.
Figure 3 (legend opposite)
2.1 \times 10^6, and 6.5 \times 10^5 clones, respectively), which identified several positive clones. One of these prey clones coded for a third RING finger protein which shares 77% and 65% homology with MURF-1 and MURF-2, respectively (Figure 3(a)). Data library searches revealed that this clone corresponded to the “MURF” described recently by Spencer et al.\(^\text{18}\) This MURF protein is referred to here as MURF-3 to distinguish it from the titin binding MURF-1 and from the MURF-2 that interacts with both MURF-1 and MURF-3 (Figure 2). Other interacting clones from this screen are currently being analyzed.

Sequence comparison of MURF-1,2,3 showed that their N-terminal 140 residues are 82% to 85% homologous and share an N-terminal RING domain, a Zn-binding B-box motif, and a MURF family-specific conserved box (MFC). Within their central regions, MURFs also share two coiled-coil dimerization motif boxes (Figures 1(c) and 3(a)). The C-terminal acidic residue-rich domains within the MURF-family are somewhat more divergent and also subject to differential splicing events (Figure 2(a)). Overall, a common feature shared by the C-terminal domains of MURFs is their high content of glutamic and aspartic amino residues (Figure 3(a)). Comparison of MURFs with other members of the RING family revealed that MURF-1,2,3 cluster together when compared with other known RING family members (Figure 3(b)). This raises the question of whether MURFs correspond to a gene cluster. When mapping the loci of MURF-1,2,3 in human, all three loci assign to different chromosomes (Table 1). Therefore, evolutionary constraints are likely to be responsible for maintaining the high degree of conservation among members of the MURF family, which is remarkably high particularly in the MURF-family specific MFC-box (Figure 3(a)).

To determine the tissue distribution of MURF transcripts, MURF-1,2,3-specific probes were hybridized to multiple tissue poly(A)\(^+\) RNA dot blots (Figure 4). MURF-1 and MURF-3 are muscle-specific, whereas low levels of MURF-2 transcripts were detected in liver in addition to striated muscles. MURF-1 transcript appeared to be expressed throughout all developmental stages in the striated muscle tissues, whereas MURF-2 transcript is most abundant in the fetal heart. MURF-3 transcript levels appeared to be upregulated during striated muscle development (Figure 4).

**Sarcomeric and cellular localization of MURF-1**

To determine the cellular localization of MURFs, antibodies were raised to MURF-1, 2 and 3 specific peptides. Immunoelectron microscopy with MURF-1 specific antibodies detected MURF epitopes 100 nm away from the center of the M-line within the periphery of the M-line lattice (Figure 5(a) and (c)). This localization coincides with that of the titin kinase domain, as determined previously with titin kinase domain specific antibodies.\(^\text{8}\) In addition to M-line labelling, MURF-1 epitopes were also detected within the periphery of the Z-line. Indirect immunofluorescence microscopy on rat heart isolated myofibrils and whole heart tissue sections detected both soluble and sarcomeric bound forms of MURF-1 (Figure 6). To determine the location of MURF-1 in the sarcomere, double immunofluorescence staining was performed with the Z line markers anti-titin T12 or anti-sarcomeric \(\alpha\)-actinin antibodies. It is interesting that in some instances, we detected most MURF-1 labelling at the M-line together with soluble forms (rat heart isolated myofibrils, Figure 6(a)). In other instances, we detected MURF-1 essentially at the Z line (rat heart tissue, Figure 6(b), and rat psoas tissue, data not shown), suggesting that MURF-1 may target to different sarcomeric as well as cytoplasmic sites. The basis for these differential localization patterns is currently under investigation.

Our antibodies raised to MURF-2 did not detect specific labeling in adult cardiac and skeletal tissues, whereas in fetal heart tissues a diffuse labelling was detected (data not shown). At present we do not know if this reflects the lack of efficient epitope recognition by our antibody, or if MURF-2 is present in soluble form in fetal tissues. Finally, MURF-3 was recently found to colocalize with microtubules and with Z-lines in skeletal muscle.\(^\text{18}\)

**Discussion**

Here, we describe the identification of three highly related RING finger proteins by titin-based yeast two-hybrid screens. One of them is identical with the recently described MURF protein.\(^\text{18}\) The three RING finger proteins discussed here represent a specific subgroup among all known RING finger proteins (Figure 3(b)). Therefore, we suggest referring to all three proteins as MURFs. To distinguish the individual members of the MURF

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**Figure 3.** Sequence analysis of the MURF family. (a) MURF-1,2,3 are highly conserved within their N-terminal 16 kDa, and share RING, B-box, MFC and CC-domains. The C termini are more divergent and also subject to differential splicing (see Figure 2(a)). Residues identical in all MURFs are colored. Yellow and red highlight Cys or His residues which are Zn-chelators; gray indicates residues with similarity. Hydrophobic residues from the conserved coiled coil heptad repeats are circled (triangles when only present in MURF-2/MURF-3). (b) Tree comparison with other members of the RING family shows that MURFs represent a specific subgroup within the RING gene superfamily.
gene family, we propose to refer to the titin binding MURF as MURF-1, to the MURF expressed early during cardiac development as MURF-2, and finally, to refer to the recently described microtubule-associated protein\textsuperscript{18} as MURF-3.

The N-terminal regions of the three MURFs contain two potential initiation codons (Figure 3(a)). To study from which ATG codon translation initiation may occur, EST entries in the public data libraries encoding the 5\textsuperscript{\textprime} regions of mouse MURFs were analyzed. A partial cDNA for mouse MURF-2 was found to be poorly conserved to human MURF-2 upstream of the 3\textsuperscript{\textprime} ATG (EST accession gb\textbackslash{}AA840584.1\textbackslash{}AA840584). In the mouse genomic sequence for MURF-1, a TATA box is located 30 base-pairs upstream of the second ATG codon (not shown). Therefore, translational initiation within the MURF family is likely to occur from the second ATG within the MURF open reading frames (see Figure 3(a)). It remains to be seen if the upstream ATG codon may also serve as a second alternative initiation site.

When searching the public databases with our human MURF-1,2,3 sequences, we noted a very high degree of conservation to MURF-like ESTs from other vertebrates (data not shown). However,

<table>
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<th>Gene locus</th>
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<th>Protein localisation</th>
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<th>Reference</th>
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<td>MURF-1, 1p31.1-p33</td>
<td>All striated muscles and developmental stages</td>
<td>Cytoplasm, M-lines, Z-lines</td>
<td>Titin-based-signal transduction?</td>
<td>This study</td>
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<tr>
<td>MURF-2, 8q12-13</td>
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<td>This study</td>
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<tr>
<td>MURF-3, 2q16-21</td>
<td>Adult striated muscle tissues. Upregulated during differentiation, differentially spliced isoforms</td>
<td>Z-lines+ microtubules</td>
<td>Microtubule stabilization</td>
<td>Spencer et al.\textsuperscript{18}</td>
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The MURF1,2, and 3 gene loci as mapped on RH-panels and their currently known biological features are listed (MURF-3: Spencer et al., 2000;\textsuperscript{18} MURF-1,2: this study).

Figure 4. Analysis of tissue-specific MURF transcription on multiple tissue RNA blots. (a) MURF-1 is expressed in all striated muscle tissues throughout development. (b) MURF-2 transcription is more restricted to cardiac tissues and appears to be downregulated during cardiac development; signals in skeletal muscles and liver are close to background. (c) MURF-3 levels increase in the adult heart when compared to fetal levels. (d) Northern blots confirm striated muscle specificity of MURF-1 and show a single ~1.9 kb full-length mRNA.
we could not identify MURF homologs in the sequenced genomes from *C. elegans* and *Drosophila melanogaster*. It will be interesting to see if yeast two-hybrid studies using the twitchin and projectin kinase N-terminal flanking domains as baits will also identify twitchin and projectin kinase domain associated proteins.

The localization of MURF-1 within the periphery of the M-line is consistent with its binding to the titin kinase region, whereas its presence in Z-lines in our study is surprising. However, recently, MURF-3 has also been localized to Z-lines. The coiled-coil potentials in MURF1/MURF-3 suggest that both molecules may form heterodimers. Indeed, our GST pulldown assays detected the interaction of MURF-1 with MURF-3 (Figure 2(c)). Therefore, we speculate that a fraction of MURF-1 targets to the M-line periphery via its interaction with titin A168/A169 sequences, whereas another fraction is bound to the Z-line by heterodimerization with MURF-3. MURF-3 in turn interacts with Z-line proteins. It is interesting that in some sections about equal amounts of M-line and Z-line bound MURF-1 were detected (Figure 5), whereas in others only Z-line bound MURF-1 was detected. This raises the interesting possibility that MURF-1 has multiple cellular localizations, with a variable, controlled distribution.

The interaction of MURF-1 both with the titin kinase flanking sequences and with MURF-3, which is involved in microtubule dynamics, may link titin-based and microtubule-based signaling pathways. For instance, it has previously been noted that the microtubule-stabilizing drug taxol induces postmitotic myoblasts to assemble interdigitating microtubule-myosin arrays, suggesting links between microtubular and sarcomeric assembly. Also, the stability and composition of microtubules in heart muscle are dependent on stretch and ventricular filling. Titin appears to be an excellent candidate for a myofibrillar-based stretch sensor, because its individual giant filamentous molecules span the entire half sarcomere, and form by their overlap in Z and M-lines a continuous filament system within the myofibril. The titin kinase domain with its associated factor(s) such as MURF-1 is therefore emerging as a potential sensor. This could link myofibril stretch to microtubule restructuring which is known to occur in pressure-induced cardiac hypertrophy.

![Figure 5](image1)

**Figure 5.** Immunoelectron microscopy of sarcomeres from mouse heart left ventricle detects MURF-1 epitopes within both the Z-lines and M-lines. (a) Electron micrograph showing Z and M-line labeling. (b) An example of peripheral-labelling of M-line. (c) Close-up view of M-line peripheral epitopes.

![Figure 6](image2)

**Figure 6.** Rat heart myofibrils and whole heart tissue sections stained with MURF-1 specific antibodies: detection of the Z-line, M-line and soluble staining patterns. (a) MURF-1 is localized to the M-line region and is also found in soluble form in rat heart isolated myofibrils. Merged double immunofluorescence confocal micrograph of MURF-1 (green) and titin T12 (red) localization in rat heart myofibrils. (b) MURF-1 was sometimes localized exclusively in the Z-line region in rat heart tissue sections. Merged double immunofluorescence confocal micrograph of MURF-1 (green) and sarcomeric α-actinin (red) localization in rat heart sections. Arrows mark MURF-1 staining.
Future studies are needed to address how the titin kinase and the MURF proteins cooperate in myofibrillar signal transduction. Potentially, the titin kinase domain may phosphorylate MURF-1, which then would operate downstream in the titin kinase signal pathway. Our preliminary in vitro studies using recombinant titin kinase and MURF-1 proteins have been unsuccessful in demonstrating specific phosphorylation of MURF-1 by titin (unpublished results). Therefore, at present, we favor a model in which MURF-1 may modulate the activity of the titin kinase domain, a domain that is tightly autoinhibited. In this model, MURF-1 may present appropriate substrates to the titin kinase domain, similar to the roles of A-protein kinase associated proteins (AKAPs) in controlling protein kinase A activities and specificities (for a review, see Dodge & Scott). Finally, it should be noted that, in many cases, RING domains mediate ubiquitin-transfer (for a review, see Dodge & Scott). This raises the interesting possibility that the association of MURFs with the M-line and Z-line regions (Figure 3) could link ubiquitin-transfer pathways to the titin filament system. At present, we are investigating whether such links exist and whether they may relate to the physiological turnover of titin in myofibrils.

Despite the discovery of a family of titin-kinase like domains in giant muscle protein and the determination of the atomic structure of the titin kinase domain the function of this catalytic domain has remained elusive. Novel insights into the function of the titin kinase signaling pathway may be derived from the present discovery and the future characterization of the MURF-1/titin kinase complex.

Materials and Methods

Yeast two-hybrid screens

The “bait” cDNA fragments were inserted into the pAS2C-1 vector (Clontech, Palo Alto), and transformed into Saccharomyces cerevisiae, strain PJ69-4A. For screening, transformed PJ69-4A cells were further transformed with human skeletal muscle, heart, brain, or mouse embryo cDNA library in the pGAD10 or pACT2 vector (Clontech, Palo Alto). Candidate clones were isolated, transformed PJ69-4A cells were further transformed with human skeletal muscle, heart, brain, or mouse embryo cDNA library in the pGAD10 or pACT2 vector (Clontech, Palo Alto). The results of the Coomassie blue stain of GST-protein in the different reactions were bound to gluthathione-Sepharose 4B beads (Amersham Pharmacia Biotech, Sweden) using approximately 100 μl of lysate on 50 μl of beads (50% slurry) in 1 ml of coating buffer (2× PBS, 1% (v/v) Triton X-100) for one hour. The beads were washed three times with coating buffer and resuspended in binding buffer (40 mM Tris (pH 7.4), 400 mM NaCl, 10% (w/v) glycerol, 0.2% (w/v) Igepal CA630). Then 8 μl of in vitro translated protein was added to 50 μl of beads coated with 20 μg of GST fusion protein in 500 μl of binding buffer. The mixture was incubated for 1.5 hours at 4°C and washed three times with binding buffer and resuspended in SDS sample buffer. The protein complexes were separated by SDS-15% PAGE. The gels were stained with Coomassie blue, treated with Amplify (Amersham Pharmacia Biotech, Sweden), dried and exposed using BioMax MR-1 film (Eastman Kodak, Rochester, NY). The results of the Coomassie blue staining were used to confirm that equal amounts of each GST-protein in the different reactions were bound to the gluthathione beads (data not shown). GST and a His6-GST fusion protein of the titin N2A region (bp73,419-74,135 (human titin cDNA, EMBL data library accession X90568) was inserted into a modified pET9d vector to express a His6-GST-MURF-1 fusion peptide. The constructs were transformed into BL21 codon plus cells (Stratagene, Heidelberg). Whole cell lysate in 2× PBS was prepared as described by Studier et al.20 GST fusion protein was immobilized on glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, Sweden) using approximately 100 μl of lysate on 50 μl of beads (50% slurry) in 1 ml of coating buffer (2× PBS, 1% (v/v) Triton X-100) for one hour. The beads were washed three times with coating buffer and resuspended in binding buffer (40 mM Tris (pH 7.4), 400 mM NaCl, 10% (w/v) glycerol, 0.2% (w/v) Igepal CA630). Then 8 μl of in vitro translated protein was added to 50 μl of beads coated with 20 μg of GST fusion protein in 500 μl of binding buffer. The mixture was incubated for 1.5 hours at 4°C and washed three times with binding buffer and resuspended in SDS sample buffer. The protein complexes were separated by SDS-15% PAGE. The gels were stained with Coomassie blue, treated with Amplify (Amersham Pharmacia Biotech, Sweden), dried and exposed using BioMax MR-1 film (Eastman Kodak, Rochester, NY). The results of the Coomassie blue staining were used to confirm that equal amounts of each GST-protein in the different reactions were bound to the gluthathione beads (data not shown). GST and a His6-GST fusion protein of the titin N2A region (bp15958-15966 of x90569) were used as control peptides.

cDNA cloning

The two-hybrid prey cDNA clones for MURF-1,2,3 corresponded to partial cDNAs, as indicated by open reading frames at their 5’ ends. Therefore, MURF-1, 2 and 3 probes were hybridized to a human cardiac cDNA library (Stratagene #956208). From a total of 400,000 screened clones, 24 positive clones were picked. Their cDNA inserts were amplified using combinations of MURF-specific internal primers with Bluescript (lambda-ZAP) derived vector primers. The largest fragments extending into the 5’ and 3’ directions were sequenced. Two or more independent fragments were sequenced for each extension to establish the correct open reading frame. For MURF-2 and MURF-3 splice isoform variants were noted within the C-terminal region (see annotations to AJ291712 and AJ291714, respectively). The human full-length cDNA sequences encoding MURF-1, 2 and 3 have been deposited in the EMBL data library under accession numbers, AJ291713, AJ291712, AJ291714, respectively.

Northern blot analysis

To determine the tissue distribution and message sizes of MURFs, MURF-1,2,3 cDNA fragments were randomly labeled with 32P (Amersham, Multiprime DNA labelling systems), and probes were hybridized to multiple tissue dot and Northern blots (Clontech, RNA Master Blot and MTN® Blot). To avoid illegitimate crosshybridization, probes were selected from the 3’ UTR regions. To
allow for a semi-quantitative comparison, three separate RNA Master Blot filters were purchased from the manufacturer, hybridized, washed and exposed to X-ray films, in parallel under identical conditions.

Gene mapping

The localization of the MURF-1,2,3 gene loci in human was determined by the radiation hybrid method on the Stanford G3 RH panel, using specific primer pairs. MURF-1 was localized to region 1p31.1-p33, 22 cR from SHGC-56764, 26 cR from SHGC-37693, and 25 rR from SHGC-19875.

MURF-2 was located 7 cR from SHGC-14626 and 14 cR from SHGC-11736. These markers reside within a 2 cM (436 cR) region between D2S171 and D2S165 on 2p21-16. MURF-2 was localized to 8q12-13, 19 cR from SHGC-12488, SHGC-3914 and SHGC-3855. SHGC-3914 localizes between markers D8S1841 and D8S1797 on the Stanford G3 map, whereas SHGC-12488 localizes between D8S1841 and D8S553 in the NCBI map. This presumably indicates discrepancies of microsatellite assignments in different chromosomal maps at this stage.

Protein expression and antibody production

For obtaining MURF-1 specific antisera, a fragment encoding its C-terminal 176 residues was amplified from total skeletal muscle cDNA by PCR, using the following primers: MURF-1S: ttctccatg GCG GGG AAT GAC CGT TGG CAG ACC ATC ATC; MURF-1R: tttggtacc TCC TTA CTG GTG TCC TTC TTC CTT. This fragment excludes the highly conserved N-terminal RING finger and MURF family box motifs, therefore reducing the risk of crossreaction to MURF-2. The fragment was cloned into the pET9D expression vector and was then extensively washed with relax-}

Immuno-electron microscopy

Isolated rat heart myofibrils or 10 μm frozen sections of adult rat heart were fixed in 2% (v/v) formaldehyde/PBS for ten minutes, washed in PBS, and permeabilized in 0.2% Triton X-100/PBS for 15 minutes. The myofibrils and sections were pre-incubated in 2% (w/v) bovine serum albumin, 1% (v/v) normal donkey serum/PBS for one hour at room temperature to minimize non-specific binding of antibodies. The sections and myofibrils were incubated with affinity purified anti-MURF-1 antibodies (10 μg/ml), followed by Texas Red-conjugated goat anti-rabbit IgG (1:700) (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). The tissue was then incubated with anti-titin T12 monoclonal antibody (1:200) (Boehringer-Mannheim Biochemicals) or with sarcomeric α-actinin monoclonal antibody (1:2000) (EA-53; Sigma; St. Louis, MO) followed by Cy-2-conjugated goat anti-mouse IgG (1:600) (Pierce; Rockford, IL). No differences in staining patterns were observed between frozen sections and isolated myofibrils. Sections stained with the secondary antibodies alone consistently revealed negligible fluorescence. Stained sections were analyzed on a Leica TCS 4D laser scanning confocal microscope (Arizona Research Laboratory, Division of Biotechnology, University of Arizona, Tucson, AZ) using a 100× NA 1.4 oil immersion objective. Simultaneous two channel recording was performed with a pinhole size of 90 μm. All Figures are derived from a single optical section. Images were processed and merged using Adobe Photoshop software and printed using a Codonics NP1600 dye sublimation printer.

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References


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